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| (71) Applicant (for all designated States except US): INNOGENETICS N.V. [BE/BE]; Industriepark Zwijnaarde 7, P.O. Box 4, B-9052 Ghent (BE).   |  |  |   |  |  |
| (72) Inventor; and  |  |  |   |  |  |
| (75) Inventor/Applicant (for US only): STUYVER, Lieven [BE/BE]; Holestraat 8, B-9552 Herzele (BE).  |  |  |   |  |  |
| (74) Common Representative: INNOGENETICS N.V.; Industriepark Zwijnaarde 7, P.O. Box 4, B-9052 Ghent (BE).   |  |  |   |  |  |
| (54) Title: METHOD FOR DETECTION OF DRUG-SELECTED MUTATIONS IN THE HIV PROTEASE GENE  |  |  |   |  |  |
| (57) Abstract   |  |  |   |  |  |
| <p>The present invention relates to a method for the rapid and reliable detection of drug-selected mutations in the HIV protease gene allowing the simultaneous characterization of a range of codons involved in drug resistance using specific sets of probes optimized to function together in a reverse-hybridization assay. More particularly, the present invention relates to a method for determining the susceptibility to antiviral drugs of HIV viruses in a biological sample, with said method comprising: a) if need be, releasing, isolating or concentrating the polynucleic acids present in the sample; b) if need be amplifying the relevant part of the protease gene of HIV with at least one suitable primer pair; c) hybridizing the polynucleic acids of step a) or b) with at least one of the following probes: probes specifically hybridizing to a target sequence comprising codon 30; probes specifically hybridizing to a target sequence comprising codon 46 and/or 48; probes specifically hybridizing to a target sequence comprising codon 50; probes specifically hybridizing to a target sequence comprising codon 54; probes specifically hybridizing to a target sequence comprising codon 82 and/or 84; probes specifically hybridizing to a target sequence comprising codon 90; <u>or the complement of said probes</u>; further characterized in that said probes specifically hybridize to any of the target sequences presented in figure (1), <u>or the complement of said target sequences</u>; d) inferring from the result of step c) whether or not a mutation giving rise to drug resistance is present in any of said target sequences.</p> |  |  |   |  |  |
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METHOD FOR DETECTION OF DRUG-SELECTED MUTATIONS  
IN THE HIV PROTEASE GENE

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1. FIELD OF THE INVENTION

The present invention relates to the field of HIV diagnosis. More particularly, the present invention relates to the field of diagnosing the susceptibility of an HIV sample to antiviral drugs used to treat HIV infection.

10 The present invention relates to a method for the rapid and reliable detection of drug-selected mutations in the HIV protease gene allowing the simultaneous characterization of a range of codons involved in drug resistance using specific sets of probes optimized to function together in a reverse-hybridization assay.

15

2. BACKGROUND OF THE INVENTION

20 The human immunodeficiency virus (HIV) is the ethiological agent for the acquired immunodeficiency syndrome (AIDS). HIV, like other retroviruses, encodes an aspartic protease that mediates the maturation of the newly produced viral particle by cleaving viral polypeptides into their functional forms (Hunter *et al.*). The HIV protease is a dimeric molecule consisting of two identical subunits each contributing a catalytic aspartic residue (Navia *et al.*, Whodawer *et al.*, Meek *et al.*). Inhibition of this enzyme gives rise to noninfectious viral particles that cannot establish new cycles of viral replication (Kohl *et al.*, Peng *et al.*).

25 Attempts to develop inhibitors of HIV-1 protease were initially based on designing peptide compounds that mimicked the natural substrate. The availability of the 3-dimensional structure of the enzyme have more recently allowed the rational design of protease inhibitors (PI) using computer modeling (Huff *et al.*, Whodawer *et al.*). A number of second generation PI that are partially peptidic or entirely nonpeptidic have proven to exhibit particularly potent antiviral effects in cell culture.

30 Combinations of various protease inhibitors with nucleoside and non-nucleoside RT inhibitors have also been studied extensively *in vitro*. In every instance, the combinations have been at least additive and usually synergistic.

35 In spite of the antiviral potency of many recently developed HIV-1 PI, the emergence of virus variants with decreased sensitivity to these compounds has been described both in cell culture and in treated patients thereby escaping the inhibitory effect of the antiviral (Condra *et al.*). Emergence of

resistant variants depends on the selective pressure applied to the viral population. In the case of a relatively ineffective drug, selective pressure is low because replication of both wild-type virus and any variants can continue. If a more effective drug suppresses replication of virus except for a resistant variant, then that variant will be selected. Virus variants that arise from selection by PI carry several 5 distinct mutations in the protease coding sequence that appear to emerge sequentially. A number of these cluster near the active site of the enzyme while others are found at distant sites. This suggests conformational adaptation to primary changes in the active site and in this respect certain mutations that increase resistance to PI also decrease protease activity and virus replication.

Amongst the PI, the antiviral activity of the PI ritonavir (A-75925; ABT-538), nelfinavir (AG-10 1343), indinavir (MK-639; L735; L524) and saquinavir (Ro 31-8959) have been approved by the Food and Drug Administration and are currently under evaluation in clinical trials involving HIV-infected patients. The VX-487 (141W94) antiviral compound is not yet approved. The most important mutations selected for the above compounds and leading to gradually increasing resistance are found at amino acid (aa) positions 30 (D to N), 46 (M to I), 48 (G to V), 50 (I to V), 54 (I to A; I to V), 82 (V to A, or F, or 15 I, or T), 84 (I to V) and 90 (L to M). Other mutations associated with drug resistance to the mentioned compounds have been described (Schinazi *et al*). Saquinavir-resistant variants, which usually carry mutations at amino acid positions 90 and/or 48, emerge in approximately 45% of patients after 1 year of monotherapy. Resistance appears to develop less frequently with higher doses of saquinavir. Resistance to indinavir and ritonavir requires multiple mutations; usually at greater than 3 and up to 11 20 sites, with more amino acid substitutions conferring higher levels of resistance. Resistant isolates usually carry mutations at codons 82, 84, or 90. In the case of ritonavir, the mutation at codon 82 appears first in most patients. Although mutant virions resistant to saquinavir are not cross-resistant to indinavir or ritonavir, isolates resistant to indinavir are generally ritonavir resistant and visa versa. Resistance to either indinavir or ritonavir usually results in cross-resistance to saquinavir. Approximately one third 25 of indinavir resistant isolates are cross-resistant to nelfinavir as well.

The regime for an efficient antiviral treatment is currently not clear at all. Patterns of reduced susceptibility to HIV protease inhibitors have been investigated *in vitro* by cultivating virus in the presence of PI. These data, however, do not completely predict the pattern of amino-acid changes actually seen in patients receiving PI. Knowledge of the resistance and cross-resistance patterns should 30 facilitate selection of optimal drug combinations and selection of sequences with non-overlapping resistance patterns. This would delay the emergence of cross-resistant viral strains and prolong the duration of effective antiretroviral activity in patients. Therefore, there is need for methods and systems that detect these mutational events in order to give a better insight into the mechanisms of HIV resistance. Further, there is need for methods and systems which can provide data important for the 35 antiviral therapy to follow in a more time-efficient and economical manner compared to the conventional cell-culture selection techniques.

### 3. AIMS OF THE INVENTION

5 It is an aim of the present invention to develop a rapid and reliable detection method for determination of the antiviral drug resistance of viruses, which contain protease genes such as HIV retroviruses present in a biological sample.

More particularly it is an aim of the present invention to provide a genotyping assay allowing the detection of the different HIV protease gene wild type and mutation codons involved in the antiviral resistance in one single experiment.

10 It is also an aim of the present invention to provide an HIV protease genotyping assay or method which allows to infer the nucleotide sequence at codons of interest and/or the amino acids at the codons of interest and/or the antiviral drug selected spectrum, and possibly also infer the HIV type or subtype isolate involved.

15 Even more particularly it is an aim of the present invention to provide a genotyping assay allowing the detection of the different HIV protease gene polymorphisms representing wild-type and mutation codons in one single experimental setup.

20 It is another aim of the present invention to select particular probes able to discriminate wild-type HIV protease sequences from mutated or polymorphic HIV protease sequences conferring resistance to one or more antiviral drugs, such as ritonavir (A-75925; ABT-538), nelfinavir (AG-1343), indinavir (MK-639; L735; L524), saquinavir (Ro 31-8959) and VX-478 (141W94) or others (Shinazi *et al*).

It is more particularly an aim of the present invention to select particular probes able to discriminate wild-type HIV protease sequences from mutated or polymorphic HIV protease sequences conferring resistance to ritonavir (A-75925; ABT-538).

25 It is more particularly an aim of the present invention to select particular probes able to discriminate wild-type HIV protease sequences from mutated HIV protease sequences conferring resistance to nelfinavir (AG-1343).

It is more particularly an aim of the present invention to select particular probes able to discriminate wild-type HIV protease sequences from mutated HIV protease sequences conferring resistance to indinavir (MK-639; L735; L524).

30 It is more particularly an aim of the present invention to select particular probes able to discriminate wild-type HIV protease sequences from mutated HIV protease sequences conferring resistance to saquinavir (Ro 31-8959).

35 It is more particularly an aim of the present invention to select particular probes able to discriminate wild-type HIV protease sequences from mutated HIV protease sequences conferring resistance to VX-478 (141W94).

It is also an aim of the present invention to select particular probes able to determine and/or infer

cross-resistance to HIV protease inhibitors.

It is more particularly an aim of the present invention to select particular probes able to discriminate wild-type HIV protease from mutated HIV protease sequences involving at least one of amino acid positions 30 (D to N), 46 (M to I), 48 (G to V), 50 (I to V), 54 (I to A or V), 82 (V to A or 5 F or I or T), 84(I to V) and 90 (L to M) of the viral protease gene.

It is particularly an aim of the present invention to select a particular set of probes, able to discriminate wild-type HIV protease sequences from mutated HIV protease sequences conferring resistance to any of the antiviral drugs defined above with this particular set of probes being used in a reverse hybridization assay.

10 It is moreover an aim of the present invention to combine a set of selected probes able to discriminate wild-type HIV protease sequences from mutated HIV protease sequences conferring resistance to antiviral drugs with another set of selected probes able to identify the HIV isolate, type or subtype present in the biological sample, whereby all probes can be used under the same hybridization and wash-conditions.

15 It is also an aim of the present invention to select primers enabling the amplification of the gene fragment(s) determining the antiviral drug resistance trait of interest.

It is also an aim of the present invention to select particular probes able to identify mutated HIV protease sequences resulting in cross-resistance to antiviral drugs.

20 The present invention also aims at diagnostic kits comprising said probes useful for developing such a genotyping assay.

The present invention also aims at diagnostic kits comprising said primers useful for developing such a genotyping assay.

#### 25 4. DETAILED DESCRIPTION OF THE INVENTION.

All the aims of the present invention have been met by the following specific embodiments.

According to one embodiment, the present invention relates to a method for determining the susceptibility to antiviral drugs of HIV viruses in a biological sample, with said method comprising:  
30 a) if need be, releasing, isolating or concentrating the polynucleic acids present in the sample;  
b) if need be amplifying the relevant part of the protease gene of HIV with at least one suitable primer pair;  
c) hybridizing the polynucleic acids of step a) or b) with at least one of the following probes:

probes specifically hybridizing to a target sequence comprising codon 30;

35 probes specifically hybridizing to a target sequence comprising codon 46 and/or 48;  
probes specifically hybridizing to a target sequence comprising codon 50;

probes specifically hybridizing to a target sequence comprising codon 54;  
probes specifically hybridizing to a target sequence comprising codon 82 and/or 84;  
probes specifically hybridizing to a target sequence comprising codon 90;  
or the complement of said probes,  
5 further characterized in that said probes specifically hybridize to any of the target sequences presented in figure 1, or to the complement of said target sequences;  
d) inferring from the result of step c) whether or not a mutation giving rise to drug resistance is present in any of said target sequences.

The numbering of HIV-1 protease gene encoded amino acids is as generally accepted in literature.

10 Mutations that give rise to an amino acid change at position 48 or 90 are known to confer resistance to saquinavir (Erlebe *et al*; Tisdale *et al*). An amino acid change at codon 46 or 54 or 82 or 84 results in ritonavir and indinavir resistance (Kempf *et al*; Emini *et al*; Condra *et al*). Amino acid changes at positions 30 and 46 confer resistance to nelfinavir (Patick *et al*) and amino acid changes at position 50 confers resistance to VX-487 (Rao *et al*). Therefore, the method described above allows to determine  
15 whether a HIV strain is susceptible or resistant to any of the drugs mentioned above. This method can be used, for instance, to screen for mutations conferring resistance to any of the mentioned drugs before initiating therapy. This method may also be used to screen for mutations that may arise during the course of therapy (i.e. monitoring of drug therapy). It is obvious that this method may also be used to determine resistance to drugs other than the above-mentioned drugs, provided that resistance to these other drugs  
20 is linked to mutations that can be detected by use of this method. This method may also be used for the specific detection of polymorphic nucleotides. It is to be understood that the said probes may only partly overlap with the targets sequences of figure 1, table 2 and table 3, as long as they allow for specific detection of the relevant polymorphic nucleotides as indicated above. The sequences of figure 1, table 2 and table 3 were derived from polynucleic acid fragments comprising the protease gene. These  
25 fragments were obtained by PCR amplification and were inserted into a cloning vector and sequence analyzed as described in example 1. It is to be noted that some polynucleic acid fragments comprised polymorphic nucleotides in their sequences, which have not been previously disclosed. These novel polymorphic nucleotide sequences are represented in table 4 below.

30 TABLE 4: Polymorphic nucleotide sequences.

|    | 51  | 52  | 53  | 54  | 55  | 56  | 57  | 58  | codon position     |  |
|----|-----|-----|-----|-----|-----|-----|-----|-----|--------------------|--|
|    | gga | ggt | ttt | atc | aaa | gta | aga | cag | consensus sequence |  |
|    | GGA | GGT | TTT | ATC | AAA | GTC | AGA | CAA | SEQ ID NO 478      |  |
| 35 | GGA | GGT | TTC | ATT | AAG | GTA | AAA | CAG | SEQ ID NO 479      |  |
|    | GGA | GGT | TTT | ATT | AAG | GTA | AGA | CAG | SEQ ID NO 480      |  |

|                                 |               |
|---------------------------------|---------------|
| GGA GGT TTT ATT AAA GTA AGA CAA | SEQ ID NO 481 |
| GGA GGC TTT ATC AAA GTA AGA CAA | SEQ ID NO 482 |
| GGA GGT TTT ATC AAA GTC AGA CAA | SEQ ID NO 483 |

|                                       |                    |
|---------------------------------------|--------------------|
| 5 78 79 80 81 82 83 84 85             | codon position     |
| gga cct aca cct gtc aac ata att gg    | consensus sequence |
| GGA CCT ACA CCG GTC AAC ATA ATT GG    | SEQ ID NO 484      |
| GGA CCT ACA CCT GCC AAT ATA ATT GG    | SEQ ID NO 485      |
| GGA CCT ACG CCC TTC AAC ATA ATT GG    | SEQ ID NO 486      |
| 10 GGA CCG ACA CCT GTC ACC ATA ATT GG | SEQ ID NO 487      |
| GGA CCT ATA CCT GTC AAC ATA ATT GG    | SEQ ID NO 488      |

|                                   |                    |
|-----------------------------------|--------------------|
| 87 88 89 90 91 92 93 94           | codon position     |
| a aga aat ctg ttg act cag att ggc | consensus sequence |
| A AAA AAT CTG ATG ACT CAG ATT GGC | SEQ ID NO 489      |
| A AGA ACT CTG TTG ACT CAG CTT GGA | SEQ ID NO 490      |
| A AGA AAT ATG ATG ACC CAG CTT GGC | SEQ ID NO 491      |
| A AGA AAT ATA ATG ACT CAG CTT GGA | SEQ ID NO 492      |
| A AGA AAT CTG CTG ACT CAG ATT GGG | SEQ ID NO 493      |
| A AGA AAT CTG TTG ACA CAG CTT GGC | SEQ ID NO 494      |
| A AGA AAT ATG TTG ACT CAG CTT GGT | SEQ ID NO 495      |
| A AGA AAT TTG TTG ACT CAG ATT GGG | SEQ ID NO 496      |
| A AGA AAT ATG TTG ACT CAG CTT GGT | SEQ ID NO 497      |
| A AGA AAT ATG TTG ACT CAG CTT GGA | SEQ ID NO 498      |
| A AGA AAT CTG TTG ACT CAG CTT GGA | SEQ ID NO 499      |
| A AGA AAC CTG TTG ACT CAA CTT GGT | SEQ ID NO 500      |

15 The present invention thus also relates to these novel sequences, or a fragment thereof, wherein said fragment consists of at least 10, preferably 15, even more preferably 20 contiguous nucleotides and contains at least one polymorphic nucleotide. It is furthermore to be understood that these new

polymorphic nucleotides may also be expected to arise in another sequence context than in the mentioned sequences. For instance a G at the third position of codon 55 is shown in SEQ ID N° 478 in combination with a T at the third position of codon 54, but a G at the third position of codon 55 may also be expected to occur in the context of a wild type sequence. It is also to be understood that the above 5 mentioned specifications apply to the complement of the said target sequences as well. This applies also to Figure 1.

According to a preferred embodiment the present invention relates to a method as indicated above, further characterized in that said probes are capable of simultaneously hybridizing to their respective target regions under appropriate hybridization and wash conditions allowing the detection of 10 the hybrids formed.

According to a preferred embodiment, step c is performed using a set of at least 2, preferably at least 3, more preferably at least 4 and most preferably at least 5 probes meticulously designed as such that they show the desired hybridization results. In general this method may be used for any purpose that relies on the presence or absence of mutations that can be detected by this method, e.g. for genotyping. 15 The probes of table 1 have been optimized to give specific hybridization results when used in a LiPA assay (see below), as described in examples 2 and 3. These probes have thus also been optimized to simultaneously hybridize to their respective target regions under the same hybridization and wash conditions allowing the detection of hybrids. The sets of probes for each of the codons 30, 46/48, 50, 54 and 82/84 have been tested experimentally as described in examples 2 and 3. The reactivity of the 20 sets shown in table 1 with 856 serum samples from various geographic origins was evaluated. It was found that the sets of probes for codons 30, 46/48, 50, 54 and 82/84 reacted with 98.9%, 99.6%, 98.5%, 99.2%, 95.4% and 97.2% of the test samples, respectively. The present invention thus also relates to the sets of probes for codons 30, 46/48, 50, 54, 82/84 and 90, shown in table 1 and table 7.

According to another even more preferred embodiment, the present invention relates to a method 25 as defined above, further characterized in that:

step b) comprises amplifying a fragment of the protease gene with at least one 5'-primer specifically hybridizing to a target sequence located between nucleotide position 210 and nucleotide position 260 (codon 87), more preferably between nucleotide position 220 and nucleotide position 260 (codon 87), more preferably between nucleotide position 230 and 30 nucleotide position 260 (codon 87), even more preferably at nucleotide position 241 to nucleotide position 260 (codon 87) in combination with at least one suitable 3'-primer, and

step c) comprises hybridizing the polynucleic acids of step a) or b) with at least one of the probes specifically hybridizing to a target sequence comprising codon 90.

35 According to another even more preferred embodiment, the present invention relates to a method as defined above, further characterized in that:

step b) comprises amplifying a fragment of the protease gene with at least one 3'-primer specifically hybridizing to a target sequence located between nucleotide position 253 (codon 85) and nucleotide positions 300, more preferably between nucleotide position 253 (codon 85) and nucleotide positions 290, more preferably between nucleotide position 253 (codon 85)

5 and nucleotide positions 280, even more preferably at nucleotide position 253 (codon 85) to nucleotide position 273 (codon 91), in combination with at least one suitable 5'-primer, and

step c) comprises hybridizing the polynucleic acids of step a) or b) with at least one of the probes specifically hybridizing to a target sequence comprising any of codons 30, 46, 48, 50, 52, 54, 82 and 84.

10 It has been found, unexpectedly, that an amplified nucleic acid fragment comprising all of the above-mentioned codons, does not hybridize optimally to probes comprising codon 82, 84 or 90. On the other hand, a shorter fragment, for instance the fragment which is amplified by use of the primers Prot41bio and Prot6bio with respectively seq id no 5 and seq id no 4, hybridizes better to probes comprising codon 90. Better hybridization is also obtained when the fragment is amplified with primer Prot41bio in

15 combination with primers Prot6abio, Prot6bbio, Prot6cbio and Prot6dbio. The present invention thus also relates to a method as defined above, further characterized in that the 5'-primer is seq id no 5 and at least one 3' primer is chosen from seq id no 4, seq id no 506, seq id no 507, seq id no 508, and seq id no 509. Likewise, another shorter fragment, for instance the fragment which is amplified by use of the primers

20 Prot2bio and Prot31bio with respectively seq id no 3 and seq id no 6, was found to hybridize better to probes comprising codon 82 and/or 84. Hence the present invention also relates to a method as defined above, further characterized in that the 5'-primer is seq id no 5 and at least one 3'-primer is chosen from seq id no 4, seq id no 506, seq id no 507, seq id no 508, and seq id no 509..

New sets of amplification primers as mentioned in example 1 were selected. The present invention thus also relates to primers: prot 16 (SEQ ID NO 501), prot 5 (SEQ ID NO 5), prot2a bio (SEQ ID NO 503),

25 prot2b bio (SEQ ID NO 504), prot31 bio (SEQ ID NO 6), prot41-bio (SEQ ID NO 505), prot6a (SEQ ID NO 506), prot6b (SEQ ID NO 507), prot6c (SEQ ID NO 508) and prot6d (SEQ ID NO 509). A number of these primers are chemically modified (biotinylated), others are not. The present invention relates to any of the primers mentioned, primers containing unmodified nucleotides, or primers containing modified nucleotides.

30 Different techniques can be applied to perform the sequence-specific hybridization methods of the present invention. These techniques may comprise immobilizing the amplified HIV polynucleic acids on a solid support and performing hybridization with labeled oligonucleotide probes. HIV polynucleic acids may also be immobilized on a solid support without prior amplification and subjected to hybridization. Alternatively, the probes may be immobilized on a solid support and hybridization may

35 be performed with labeled HIV polynucleic acids, preferably after amplification. This technique is called reverse hybridization. A convenient reverse hybridization technique is the line probe assay (LiPA). This

assay uses oligonucleotide probes immobilized as parallel lines on a solid support strip (Stuyver et al., 1993). It is to be understood that any other technique based on the above-mentioned methods is also covered by the present invention.

According to another preferred embodiment, the present invention relates to any of the probes mentioned above and/or to any of the primers mentioned above, with said primers and probes being designed for use in a method for determining the susceptibility to antiviral drugs of HIV viruses in a sample. According to an even more preferred embodiment, the present invention relates to the probes with seq id no 7 to seq id no 477 and seq id no 510 to seq id no 519, more preferably to the seq id no mentioned in Table 1 and Table 7, and to the primers with seq id no 3, 4, 5 and 6, 501, 502, 503, 504, 505, 506, 507, 508 and 509. The skilled man will recognize that addition or deletion of one or more nucleotides at their extremities may adapt the said probes and primers. Such adaptations may be required if the conditions of amplification or hybridization are changed, or if the amplified material is RNA instead of DNA, as is the case in the NASBA system.

According to another preferred embodiment, the present invention relates to a diagnostic kit enabling a method for determining the susceptibility to antiviral drugs of HIV viruses in a biological sample, with said kit comprising:

- a) when appropriate, a means for releasing, isolating or concentrating the polynucleic acids present in said sample;
- b) when appropriate, at least one of the primers of any of claims 4 to 6;
- c) at least one of the probes of any of claims 1 to 3, possibly fixed to a solid support;
- d) a hybridization buffer, or components necessary for producing said buffer;
- e) a wash solution, or components necessary for producing said solution;
- f) when appropriate, a means for detecting the hybrids resulting from the preceding hybridization;
- h) when appropriate, a means for attaching said probe to a solid support.

## 25 DEFINITIONS

The following definitions serve to illustrate the terms and expressions used in the present invention.

The term "antiviral drugs" refers particularly to any antiviral protease inhibitor. Examples of such antiviral drugs and the mutation they may cause in the HIV protease gene are disclosed in Schinazi et al., 1997. The contents of the latter two documents particularly are to be considered as forming part 30 of the present invention. The most important antiviral drugs focussed at in the present invention are disclosed in Tables 1 to 2.

The target material in the samples to be analyzed may either be DNA or RNA, e.g.: genomic DNA, messenger RNA, viral RNA or amplified versions thereof. These molecules are also termed polynucleic acids.

35 It is possible to use genomic DNA or RNA molecules from HIV samples in the methods according to the present invention.

Well-known extraction and purification procedures are available for the isolation of RNA or DNA from a sample (fi. in Maniatis et al., Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbour Laboratory Press (1989)).

The term "probe" refers to single stranded sequence-specific oligonucleotides, which have a sequence, which is complementary to the target sequence to be detected.

The term "target sequence" as referred to in the present invention describes the wild type nucleotide sequence, or the sequence comprising one or more polymorphic nucleotides of the protease gene to be specifically detected by a probe according to the present invention. This nucleotide sequence may encompass one or several nucleotide changes. Target sequences may refer to single nucleotide positions, codon positions, nucleotides encoding amino acids or to sequences spanning any of the foregoing nucleotide positions. In the present invention said target sequence often includes one or two variable nucleotide positions.

The term "polymorphic nucleotide" indicates a nucleotide in the protease gene of a particular HIV virus that is different from the nucleotide at the corresponding position in at least one other HIV virus. The polymorphic nucleotide may or may not give rise to resistance to an antiviral drug.

It is to be understood that the complement of said target sequence is also a suitable target sequence in some cases. The target sequences as defined in the present invention provide sequences which should be complementary to the central part of the probe which is designed to hybridize specifically to said target region.

The term "complementary" as used herein means that the sequence of the single stranded probe is exactly the (inverse) complement of the sequence of the single-stranded target, with the target being defined as the sequence where the mutation to be detected is located.

"Specific hybridization" of a probe to a target sequence of the HIV polynucleic acids means that said probe forms a duplex with part of this region or with the entire region under the experimental conditions used, and that under those conditions said probe does not form a duplex with other regions of the polynucleic acids present in the sample to be analyzed.

Since the current application requires the detection of single basepair mismatches, very stringent conditions for hybridization are required, allowing in principle only hybridization of exactly complementary sequences. However, variations are possible in the length of the probes (see below), and it should be noted that, since the central part of the probe is essential for its hybridization characteristics, possible deviations of the probe sequence versus the target sequence may be allowable towards head and tail of the probe, when longer probe sequences are used. These variations, which may be conceived from the common knowledge in the art, should however always be evaluated experimentally, in order to check if they result in equivalent hybridization characteristics than the exactly complementary probes.

Preferably, the probes of the invention are about 5 to 50 nucleotides long, more preferably from about 10 to 25 nucleotides. Particularly preferred lengths of probes include 10, 11, 12, 13, 14, 15, 16, 17,

18, 19, 20, 21, 22, 23, 24 or 25 nucleotides. The nucleotides as used in the present invention may be ribonucleotides, deoxyribonucleotides and modified nucleotides such as inosine or nucleotides containing modified groups, which do not essentially alter their hybridization characteristics.

5 Probe sequences are represented throughout the specification as single stranded DNA oligonucleotides from the 5' to the 3' end. It is obvious to the man skilled in the art that any of the below-specified probes can be used as such, or in their complementary form, or in their RNA form (wherein U replaces T).

10 The probes according to the invention can be prepared by cloning of recombinant plasmids containing inserts including the corresponding nucleotide sequences, if need be by cleaving the latter out from the cloned plasmids upon using the adequate nucleases and recovering them, e.g. by fractionation according to molecular weight. The probes according to the present invention can also be synthesized chemically, for instance by the conventional phospho-triester method.

15 The term "solid support" can refer to any substrate to which an oligonucleotide probe can be coupled, provided that it retains its hybridization characteristics and provided that the background level of hybridization remains low. Usually the solid substrate will be a microtiter plate, a membrane (e.g. nylon or nitrocellulose) or a microsphere (bead) or a chip. Prior to application to the membrane or fixation it may be convenient to modify the nucleic acid probe in order to facilitate fixation or improve the hybridization efficiency. Such modifications may encompass homopolymer tailing, coupling with different reactive groups such as aliphatic groups, NH<sub>2</sub> groups, SH groups, carboxylic groups, or 20 coupling with biotin, haptens or proteins.

25 The term "labeled" refers to the use of labeled nucleic acids. Labeling may be carried out by the use of labeled nucleotides incorporated during the polymerase step of the amplification such as illustrated by Saiki et al. (1988) or Bej et al. (1990) or labeled primers, or by any other method known to the person skilled in the art. The nature of the label may be isotopic (<sup>32</sup>P, <sup>35</sup>S, etc.) or non-isotopic (biotin, digoxigenin, etc.).

30 The term "primer" refers to a single stranded oligonucleotide sequence capable of acting as a point of initiation for synthesis of a primer extension product, which is complementary to the nucleic acid strand to be copied. The length and the sequence of the primer must be such that they allow to prime the synthesis of the extension products. Preferably the primer is about 5-50 nucleotides long. Specific length and sequence will depend on the complexity of the required DNA or RNA targets, as well as on the conditions of primer use such as temperature and ionic strength.

35 The term "primer pair" refers to a set of primers comprising at least one 5' primer and one 3' primer. The primer pair may consist of more than two primers, the complexity of the number of primers will depend on the hybridization conditions, variability of the sequences in the regions to be amplified and the target sequences to be detected.

The fact that amplification primers do not have to match exactly with the corresponding template

sequence to warrant proper amplification is amply documented in the literature (Kwok et al., 1990).

The amplification method used can be either polymerase chain reaction (PCR; Saiki et al., 1988), ligase chain reaction (LCR; Landgren et al., 1988; Wu & Wallace, 1989; Barany, 1991), nucleic acid sequence-based amplification (NASBA; Guatelli et al., 1990; Compton, 1991), transcription-based amplification system (TAS; Kwok et al., 1989), strand displacement amplification (SDA; Duck, 1990) or amplification by means of Q $\beta$  replicase (Lomeli et al., 1989) or any other suitable method to amplify nucleic acid molecules known in the art.

The oligonucleotides used as primers or probes may also comprise nucleotide analogues such as phosphorothioates (Matsukura et al., 1987), alkylphosphorothioates (Miller et al., 1979) or peptide nucleic acids (Nielsen et al., 1991; Nielsen et al., 1993) or may contain intercalating agents (Asseline et al., 1984).

As most other variations or modifications introduced into the original DNA sequences of the invention these variations will necessitate adaptations with respect to the conditions under which the oligonucleotide should be used to obtain the required specificity and sensitivity. However the eventual results of hybridization will be essentially the same as those obtained with the unmodified oligonucleotides.

The introduction of these modifications may be advantageous in order to positively influence characteristics such as hybridization kinetics, reversibility of the hybrid-formation, biological stability of the oligonucleotide molecules, etc.

The "sample" may be any biological material taken either directly from the infected human being (or animal), or after culturing (enrichment). Biological material may be e.g. expectorations of any kind, broncheolavages, blood, skin tissue, biopsies, sperm, lymphocyte blood culture material, colonies, liquid cultures, fecal samples, urine etc.

The sets of probes of the present invention will include at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 25 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more probes. Said probes may be applied in two or more distinct and known positions on a solid substrate. Often it is preferable to apply two or more probes together in one and the same position of said solid support.

For designing probes with desired characteristics, the following useful guidelines known to the person skilled in the art can be applied.

Because the extent and specificity of hybridization reactions such as those described herein are affected by a number of factors, manipulation of one or more of those factors will determine the exact sensitivity and specificity of a particular probe, whether perfectly complementary to its target or not. The importance and effect of various assay conditions, explained further herein, are known to those skilled in the art.

The stability of the [probe : target] nucleic acid hybrid should be chosen to be compatible with the assay conditions. This may be accomplished by avoiding long AT-rich sequences, by terminating the

hybrids with G;C base pairs, and by designing the probe with an appropriate Tm. The beginning and end points of the probe should be chosen so that the length and %GC result in a Tm about 2-10°C higher than the temperature at which the final assay will be performed. The base composition of the probe is significant because G-C base pairs exhibit greater thermal stability as compared to A-T base pairs due 5 to additional hydrogen bonding. Thus, hybridization involving complementary nucleic acids of higher G-C content will be stable at higher temperatures.

Conditions such as ionic strength and incubation temperature under which a probe will be used should also be taken into account when designing a probe. It is known that hybridization will increase as the ionic strength of the reaction mixture increases, and that the thermal stability of the hybrids will 10 increase with increasing ionic strength. On the other hand, chemical reagents, such as formamide, urea, DMSO and alcohols, which disrupt hydrogen bonds, will increase the stringency of hybridization. Destabilization of the hydrogen bonds by such reagents can greatly reduce the Tm. In general, optimal hybridization for synthetic oligonucleotide probes of about 10-50 bases in length occurs approximately 5°C below the melting temperature for a given duplex. Incubation at temperatures below the optimum 15 may allow mismatched base sequences to hybridize and can therefore result in reduced specificity.

It is desirable to have probes, which hybridize only under conditions of high stringency. Under high stringency conditions only highly complementary nucleic acid hybrids will form; hybrids without a sufficient degree of complementarity will not form. Accordingly, the stringency of the assay conditions determines the amount of complementarity needed between two nucleic acid strands forming a hybrid. 20 The degree of stringency is chosen such as to maximize the difference in stability between the hybrid formed with the target and the nontarget nucleic acid. In the present case, single base pair changes need to be detected, which requires conditions of very high stringency.

The length of the target nucleic acid sequence and, accordingly, the length of the probe sequence can also be important. In some cases, there may be several sequences from a particular region, varying 25 in location and length, which will yield probes with the desired hybridization characteristics. In other cases, one sequence may be significantly better than another that differs merely by a single base. While it is possible for nucleic acids that are not perfectly complementary to hybridize, the longest stretch of perfectly complementary base sequence will normally primarily determine hybrid stability. While oligonucleotide probes of different lengths and base composition may be used, preferred oligonucleotide 30 probes of this invention are between about 5 to 50 (more particularly 10-25) bases in length and have a sufficient stretch in the sequence which is perfectly complementary to the target nucleic acid sequence.

Regions in the target DNA or RNA, which are known to form strong internal structures inhibitory to hybridization, are less preferred. Likewise, probes with extensive self-complementarity should be avoided. As explained above, hybridization is the association of two single strands of 35 complementary nucleic acids to form a hydrogen bonded double strand. It is implicit that if one of the two strands is wholly or partially involved in a hybrid that it will be less able to participate in formation

of a new hybrid. There can be intramolecular and intermolecular hybrids formed within the molecules of one type of probe if there is sufficient self complementarity. Such structures can be avoided through careful probe design. By designing a probe so that a substantial portion of the sequence of interest is single stranded, the rate and extent of hybridization may be greatly increased. Computer programs are 5 available to search for this type of interaction. However, in certain instances, it may not be possible to avoid this type of interaction.

Standard hybridization and wash conditions are disclosed in the Materials & Methods section of the Examples. Other conditions are for instance 3X SSC (Sodium Saline Citrate), 20% deionized FA (Formamide) at 50°C.

10 Other solutions (SSPE (Sodium saline phosphate EDTA), TMACl (Tetramethyl ammonium Chloride), etc.) and temperatures can also be used provided that the specificity and sensitivity of the probes is maintained. If need be, slight modifications of the probes in length or in sequence have to be carried out to maintain the specificity and sensitivity required under the given circumstances.

15 Primers may be labeled with a label of choice (e.g. biotin). Different primer-based target amplification systems may be used, and preferably PCR-amplification, as set out in the examples. Single-round or nested PCR may be used.

The term "hybridization buffer" means a buffer enabling a hybridization reaction to occur between the probes and the polynucleic acids present in the sample, or the amplified products, under the appropriate stringency conditions.

20 The term "wash solution" means a solution enabling washing of the hybrids formed under the appropriate stringency conditions.

The following examples only serve to illustrate the present invention. These examples are in no way intended to limit the scope of the present invention.

## 25 FIGURE AND TABLE LEGENDS

Figure 1: Natural and drug selected variability in the vicinity of codons 30, 46, 48, 50, 54, 82, 84, and 90 of the HIV-1 protease gene. The most frequently observed wild-type sequence is shown in the top line. Naturally occurring variations are indicated below and occur independently from each other.

30 Drug-selected variants are indicated in bold

Figure 2 A: Reactivities of the selected probes for codon 30 immobilized on LiPA strips with reference material. The information in the boxed surface is not relevant for the discussion of probes for codon 30. The position of each selected probe on the membrane strip is shown at the left of each panel. The 35 sequence of the relevant part of the selected probes is shown at the left and is given in Table 1. Each strip is incubated with a biotinylated PCR fragment from the reference panel. The reference panel accession

numbers are indicated in Table 1. For several probes multiple reference panel possibilities are available, but only one relevant accession number given each time. \*: False positive reactivities. At the bottom the strips, the amino acids at the relevant codon, as derived from the probe reactivity, is indicated.

5 Figure 2 B: Reactivities of the selected probes for codons 46 and 48 immobilized on LiPA strips with reference material. The information in the boxed surface is not relevant for the discussion of probes for condons 46 and 48. The position of each selected probe on the membrane strip is shown at the left of each panel. The sequence of the relevant part of the selected probes is given in Table 1. Each strip is incubated with a biotinylated PCR fragment from the reference panel. The reference panel accession numbers are  
10 indicated in Table 1. For several probes multiple reference panel possibilities are available, but only one relevant accession number given each time. \*: False positive reactivities. On top of the strips, the amino acids at the relevant codon, as derived from the probe reactivity, is indicated.

15 Figure 2 C: Reactivities of the selected probes for codon 50 immobilized on LiPA strips with reference material. The information in the boxed surface is not relevant for the discussion of probes for condon 50. The position of each selected probe on the membrane strip is shown at the left of each panel. The sequence of the relevant part of the selected probes is given in Table 1. Each strip is incubated with a biotinylated PCR fragment from the reference panel. The reference panel accession numbers are indicated in Table 1. For several probes multiple reference panel possibilities are available, but only one relevant accession number given each time. \*: False positive reactivities. At the bottom of the strips, the amino acids at the relevant codon, as derived from the probe reactivity, is indicated.

20 Figure 2 D: Reactivities of the selected probes for codon 54 immobilized on LiPA strips with reference material. The information in the boxed surface is not relevant for the discussion of probes for condon 54. The position of each selected probe on the membrane strip is shown at the left of each panel. The sequence of the relevant part of the selected probes is given in Table 1. Each strip is incubated with a biotinylated PCR fragment from the reference panel. The reference panel accession numbers are indicated in Table 1. For several probes multiple reference panel possibilities are available, but only one relevant accession number given each time. \*: False positive reactivities. At the bottom of the strips, the amino acids at the relevant codon, as derived from the probe reactivity, is indicated.

25 Figure 2 E.: Reactivities of the selected probes for codons 82 and 84 immobilized on LiPA strips with reference material. The information in the boxed surface is not relevant for the discussion of probes for condons 82 and 84. The position of each selected probe on the membrane strip is shown at the left of each panel. The sequence of the relevant part of the selected probes is given in Table 1. Each strip is incubated with a biotinylated PCR fragment from the reference panel. The reference panel accession

numbers are indicated in Table 1. For several probes multiple reference panel possibilities are available, but only one relevant accession number given each time. \*: False positive reactivities. At the bottom of the strips, the amino acids at the relevant codon, as derived from the probe reactivity, is indicated.

5 Figure 2 F: Reactivities of the selected probes for codon 90 immobilized on LiPA strips with reference material. The information in the boxed surface is not relevant for the discussion of probes for codon 90. The position of each selected probe on the membrane strip is shown at the left of each panel. The sequence of the relevant part of the selected probes is given in Table 1. Each strip is incubated with a biotinylated PCR fragment from the reference panel. The reference panel accession numbers are  
10 indicated in Table 1. For several probes multiple reference panel possibilities are available, but only one relevant accession number given each time. \*: False positive reactivities. At the bottom of the strips, the amino acids at the relevant codon, as derived from the probe reactivity, is indicated.

Figure 3: Sequence and position of the HIV-1 protease amplification primers. To obtain the reactivity  
15 with probes selected to determine the susceptibility to antiviral drugs involving codons 30, 46, 48, 50, 54, 82, and 84, nested amplification primers prot2bio(5' primer) and Prot31bio (3' primer) were designed. To obtain the reactivity with probes selected to determine the susceptibility to antiviral drugs involving codon 90, nested amplification primers Prot41bio (5' primer) and Prot6bio (3' primer) were designed.

20 Figure 4 A: Phylogenetic analysis on 312 protease sequences allowed to separate genotype B strains from non-B strains. Reactivities of the selected probes for codon 30 immobilized on LiPA strips with a biotinylated PCR fragment of genotype B strains and non-B strains is shown, the exact percentages are indicated in table 5. The probes are indicated at the bottom. The sequence of the relevant part of the probes is given in Table 1.

25 Figure 4 B: Phylogenetic analysis on 312 protease sequences allowed to separate genotype B strains from non-B strains. Reactivities of the selected probes for codons 46/48 immobilized on LiPA strips with a biotinylated PCR fragment of genotype B strains and non-B strains is shown, the exact percentages are indicated in table 5. The probes are indicated at the bottom. The sequence of the relevant part of the  
30 probes is given in Table 1.

Figure 4 C: Phylogenetic analysis on 312 protease sequences allowed to separate genotype B strains from non-B strains. Reactivities of the selected probes for codon 50 immobilized on LiPA strips with a biotinylated PCR fragment of genotype B strains and non-B strains is shown, the exact percentages are indicated in table 5. The probes are indicated at the bottom. The sequence of the relevant part of the  
35 probes is given in Table 1.

5 Figure 4 D: Phylogenetic analysis on 312 protease sequences allowed to separate genotype B strains from non-B strains. Reactivities of the selected probes for codon 54 immobilized on LiPA strips with a biotinylated PCR fragment of genotype B strains and non-B strains is shown, the exact percentages are indicated in table 5. The probes are indicated at the bottom. The sequence of the relevant part of the probes is given in Table 1.

10 Figure 4 E: Phylogenetic analysis on 312 protease sequences allowed to separate genotype B strains from non-B strains. Reactivities of the selected probes for codons 82/84 immobilized on LiPA strips with a biotinylated PCR fragment of genotype B strains and non-B strains is shown, the exact percentages are indicated in table 5. The probes are indicated at the bottom. The sequence of the relevant part of the probes is given in Table 1.

15 Figure 4 F: Phylogenetic analysis on 312 protease sequences allowed to separate genotype B strains from non-B strains. Reactivities of the selected probes for codon 90 immobilized on LiPA strips with a biotinylated PCR fragment of genotype B strains and non-B strains is shown, the exact percentages are indicated in table 5. The probes are indicated at the bottom. The sequence of the relevant part of the probes is given in Table 1.

20 Figure 5 A: Geographical origin of 856 samples and reactivities with the different probes at codon position 30. The exact percentages are indicated in table 6. The probes are indicated at the bottom.

25 Figure 5 B: Geographical origin of 856 samples and reactivities with the different probes at codon positions 46/48. The exact percentages are indicated in table 6. The probes are indicated at the bottom.

Figure 5 C: Geographical origin of 856 samples and reactivities with the different probes at codon position 50. The exact percentages are indicated in table 6. The probes are indicated at the bottom.

30 Figure 5 D: Geographical origin of 856 samples and reactivities with the different probes at codon position 54. The exact percentages are indicated in table 6. The probes are indicated at the bottom.

Figure 5 E: Geographical origin of 856 samples and reactivities with the different probes at codon positions 82/84. The exact percentages are indicated in table 6. The probes are indicated at the bottom.

35 Figure 5 F: Geographical origin of 856 samples and reactivities with the different probes at codon position 90. The exact percentages are indicated in table 6. The probes are indicated at the bottom.

5 Table 1: HIV-1 protease wild-type and drug-selected mutation probes with their corresponding sequences as applied on the HIV-1 protease LiPA strip. The most frequently observed wild-type sequence is shown at the top line. Probe names corresponding to the selected motifs are indicated in the left column, the relevant part of each probe applied on the strip is shown under the consensus sequence.

Table 2: Protease Inhibitors.

10 Table 3: HIV-1 protease wild-type and drug-selected mutation probes with their corresponding sequences as synthesized, immobilized and tested on LiPA strips. The most frequently observed wild-type sequence is shown at the top line. Probe names corresponding to the selected motifs are indicated in the left column, the relevant part of each probe applied on the strip is shown under the consensus sequence. The probes retained are indicated in table 1.

15 Table 4: Polymorphic nucleotide sequences.

Table 5: % Reactivities of the HIV-1 protease wild-type and drug-selected mutation probes applied on the HIV-1 protease LiPA strip with genotype B strains and non-B strains.

20 Table 6: % Reactivities of the HIV-1 protease wild-type and drug-selected mutation probes applied on the HIV-1 protease LiPA strip with samples of different geographical origin.

25 Table 7: HIV-1 protease wild-type and drug-selected mutation probes with their corresponding sequences as applied on the HIV-1 protease LiPA strip. The most frequently observed wild-type sequence is shown at the top line. Probe names corresponding to the selected motifs are indicated in the left column, the relevant part of each probe applied on the strip is shown under the consensus sequence.

**EXAMPLES**

30 **Example 1:**

**Selection of the plasma samples, PCR amplification and cloning of the PCR products.**

35 Plasma samples (n=557) were taken from HIV type-1 infected patients and stored at -20°C until use. Plasma samples were obtained from naive and drug-treated patients. The drugs involved ritonavir, indinavir and saquinavir. The serum samples were collected from patients residing in Europe (Belgium, Luxembourg, France, Spain and UK), USA and Brazil.

HIV RNA was prepared from these samples using the guanidinium-phenol procedure. Fifty  $\mu$ l plasma was mixed with 150  $\mu$ l Trizol<sup>®</sup>LS Reagent (Life Technologies, Gent, Belgium) at room temperature (volume ratio: 1unit sample/ 3 units Trizol ). Lysis and denaturation occurred by carefully pipetting up and down several times, followed by an incubation step at room temperature for at least 5 minutes. Fourty  $\mu$ l CHCl<sub>3</sub> was added and the mixture was shaken vigorously by hand for at least 15 seconds, and incubated for 15 minutes at room temperature. The samples were centrifuged at maximum 12,000g for 15 minutes at 4°C, and the colorless aqueous phase was collected and mixed with 100  $\mu$ l isopropanol. To visualize the minute amounts of viral RNA, 20  $\mu$ l of 1 $\mu$ g/ $\mu$ l Dextran T500 (Pharmacia) was added, mixed and left at room temperature for 10 minutes. Following centrifugation at max. 12,000g for 10 minutes at 4°C and aspiration of the supernatant, the RNA pellet was washed with 200  $\mu$ l ethanol, mixed by vortexing and collected by centrifugation at 7,500g for 5 minutes at 4°C. Finally the RNA pellet was briefly air-dried and stored at -20°C. Alternatively, the High Pure Viral Nucleic Acid Kit (Boehringer Mannheim ) was used to extract RNA from the samples

For cDNA synthesis and PCR amplification, the RNA pellet was dissolved in 15  $\mu$ l random primers (20 ng/ $\mu$ l, pdN<sub>6</sub>, Pharmacia), prepared in DEPC-treated or HPLC grade water. After denaturation at 70°C for 10 minutes, 5  $\mu$ l cDNA mix was added, composed of 4  $\mu$ l 5x AMV-RT buffer (250mM Tris.HCl pH 8.5, 100mM KCl, 30mM MgCl<sub>2</sub>, 25 mM DTT), 0.4  $\mu$ L 25mM dXTPs, 0.2  $\mu$ l or 25U Ribonuclease Inhibitor (HPRI, Amersham), and 0.3  $\mu$ l or 8U AMV-RT (Stratagene). cDNA synthesis occurred during the 90 minutes incubation at 42°C. The HIV -1 protease gene was than amplified using the following reaction mixture: 5  $\mu$ l cDNA, 4.5  $\mu$ l 10x Taq buffer, 0.3  $\mu$ l 25 mM dXTPs, 1  $\mu$ l (10 pmol) of each PCR primer, 38  $\mu$ l H<sub>2</sub>O, and 0.2  $\mu$ l (1 U) Taq. . Alternatively, the Titon One Tube RT-PCR system (Boehringer Mannheim) was used to perform RT-PCR.

Codon positions involving resistance to saquinavir, ritonavir, indinavir, nelfinavir and VX-478 have been described (Shinazi *et al*) and PCR amplification primers were chosen outside these regions. The primer design was based on HIV-1 published sequences (mainly genotype B clade) (Myers *et al.*) and located in regions that showed a high degree of nucleotide conservation between the different HIV-1 clades. The final amplified region covered the HIV-1 protease gene from codon 9 to codon 99. The primers for amplification had the following sequence: outer sense primer Pr16: 5' bio-CAGAGCCAACAGCCCCACCAG3' (SEQ ID NO 1); nested sense primer Prot 2 bio: 5' CCT CAR ATC 30 ACT CTT TGG CAA CG 3' (SEQ ID NO 3) ; nested antisense primer Prot 6 bio: 3' TAA TCR GGA TAA CTY TGA CAT GGT C 5' (SEQ ID NO 4); and outer antisense primer RT12: 5' bioATCAGGATGGAGTTCATACCCATCCA3' (SEQ ID NO 2). Annealing occurred at 57°C, extension at 72°C and denaturation at 94°C. Each step of the cycle took 1 minute, the outer PCR contained 40 cycles, the nested round 35. Nested round PCR products were analyzed on agarose gel and only clearly visible amplification products were used in the LiPA procedure. Quantification of viral

RNA was obtained with the HIV Monitor<sup>TM</sup> test (Roche, Brussels, Belgium). Later on, new sets of primers for amplification were selected. For the amplification of HIV protease codon 30-84: outer sense primer prot16: 5'-CAGAGCCAACAGCCCCACCAG-3' (SEQ ID NO 501), outer antisense primer prot5: 5'-TTTTCTTCTGTCAATGGCCATTGTTT-3' (SEQ ID NO 502) were used. Annealing occurred at 50°C, extension at 68°C and denaturation at 94°C for 35 cycles for the outer PCR. For the nested PCR annealing occurred at 45°C, denaturation at 94°C and extension at 92°C with primers: nested sense primers prot2a-bio: 5'-bio-CCTCAAATCACTCTTGGCAACG-3' (SEQ ID NO 503) and prot2b-bio: 5'-bio-CCTCAGATCACTCTTGGCAACG-3' (SEQ ID NO 504), and nested antisense primer prot31-bio: 5'-bio-AGTCAACAGATTCTTCCAAT-3' (SEQ ID NO 6). For the amplification of HIV protease codon 90, the outer PCR was as specified for HIV protease codon 30-84. For the nested PCR, nested sense primer prot41-bio: 5'-bio-CCTGTCAACATAATTGCAAG-3' (SEQ ID NO 505) and nested antisense primers prot6a: 5'-bio-CTGGTACAGTTCAATAGGACTAAT-3' (SEQ ID NO 506), prot6b: 5'-bio-CTGGTACAGTCTCAATAGGACTAAT-3' (SEQ ID NO 507), prot6c: 5'-bio-CTGGTACAGTCTCAATAGGACTAAT-3' (SEQ ID NO 508), prot6d: 5'-bio-CTGGTACAGTCTCAATAGGACTAAT-3' (SEQ ID NO 509) were used. For the nested PCR the annealing temperature occurred at 45°C. Primers were tested on a plasmid, which contained an HIV fragment of 1301 bp ligated in a pGEM-T vector. The fragment contains protease, reverse transcriptase and the primer sites of first and second round PCR. By restriction with *Sac I* the plasmid is linearised.

Selected PCR products were cloned into the pretreated EcoRV site of the pGEMT vector (Promega). Recombinant clones were selected after  $\alpha$ -complementation and restriction fragment length analysis, and sequenced using standard sequencing techniques with plasmid primers and internal HIV protease primers. Sometimes biotinylated fragments were directly sequenced with a dye-terminator protocol (Applied Biosystems) using the amplification primers. Alternatively, nested PCR was carried out with analogs of the nested primers, in which the biotin group was replaced with the T7- and SP6-primer sequence, respectively. These amplicons were then sequenced with an SP6- and T7-dye-primer procedure.

#### Example 2:

##### 30 Selection of a reference panel

Codon positions involving resistance to saquinavir, ritonavir, indinavir, nelfinavir and VX-478 have been described (Shinazi *et al.* 1997). It was the aim to clone in plasmids those viral protease genes that are covering the different genetic motifs at those important codon positions conferring resistance against the described protease inhibitors.

35 After careful analysis of 312 protease gene sequences, obtained after direct sequencing of PCR fragments, a selection of 47 PCR fragments which covered the different target polymorphisms and

mutations were retained and cloned in plasmids using described cloning techniques. The selection of samples originated from naive or drug-treated European, Brazilian or US patients. These 47 recombinant plasmids are used as a reference panel, a panel that was sequenced on both strands, and biotinylated PCR products from this panel were used to optimize probes for specificity and sensitivity.

5 Although this panel of 47 samples is a representative selection of clones at this moment, it is important to mention here that this selection is in fact only a temporally picture of the variability of the virus, and a continuous update of this panel will be mandatory. This includes on ongoing screening for the new variants of the virus, and recombinant cloning of these new motifs.

10 **Probe selection and LiPA testing.**

To cover all the different genetic motifs in the reference panel, a total of 471 probes were designed (codon 30: 40 probes; codon 46/48: 72 probes; codon 50:55 probes; codon 54: 54 probes; codon 82/84: 130 probes; codon 90: 120 probes). Table 3 shows the different probes that were selected for the different codon positions.

15 It was the aim to adapt all probes to react specifically under the same hybridization and wash conditions by carefully considering the % (G+C), the probe length, the final concentration of the buffer components, and hybridization temperature (Stuyver et al., 1997). Therefore, probes were provided enzymatically with a poly-T-tail using the TdT (Pharmacia) in a standard reaction condition, and purified via precipitation. For a limited number of probes with 3' T-ending sequences, an additional G was  
20 incorporated between the probe sequence and the poly-T-tail in order to limit the hybridizing part to the specific probe sequence and to exclude hybridization with the tail sequence. Probe pellets were dissolved in standard saline citrate (SSC) buffer and applied as horizontal parallel lines on a membrane strip. Control lines for amplification (probe 5' TAGGGGAAATTGGAGGTTTAG 3', HIV protease aa 47 to aa 54) and conjugate incubation (biotinylated DNA) were applied alongside. Probes were  
25 immobilized onto membranes by baking, and the membranes were sliced into 4mm strips also called LiPA strips.

Selection of the amplification primers and PCR amplification was as described in example 1. In order to select specific reacting probes out of the 471 candidate probes, LiPA tests were performed with biotinylated PCR fragments from the reference panel. To perform LiPA tests, equal amounts (10  
30  $\mu$ l) of biotinylated amplification products and denaturation mixture (0.4 N NaOH/0.1% SDS) were mixed, followed by an incubation at room temperature for 5 minutes. Following this denaturation step, 2 ml hybridization buffer (2xSSC, 0.1% SDS, 50mM Tris pH7.5) was added together with a membrane strip and hybridization was carried out at 39°C for 30 min. Then, the hybridization mixture was replaced by stringent washing buffer (same composition as hybridization buffer), and stringent washing occurred  
35 first at room temperature for 5 minutes and then at 39°C for another 25 minutes. Buffers were then replaced to be suitable for the streptavidine alkaline phosphatase conjugate incubations. After 30 minutes

incubation at room temperature, conjugate was rinsed away and replaced by the substrate components for alkaline phosphatase, Nitro-Blue-Tetrazolium and 5-Bromo-4-Chloro-3-Indolyl Phosphate. After 30 minutes incubation at room temperature, probes where hybridization occurred became visible because of the purple brown precipitate at these positions.

5 After careful analysis of the 471 probes, the most specific and sensitive probes (n=46) were finally selected, covering the natural and drug-selected variability in the vicinity of aa. 30, 46, 48, 50, 54, 82, 84, and 90. Figure 2 shows the reactivity of the finally selected probes with the reference panel.

**Example 3:**

10 **LiPA testing on clinical samples.**

A total of 856 samples were tested on this selection of 46 specific probes. The geographical origin of these samples is as follows: USA:359 ; France: 154; UK:36; Brazil 58; Spain 35; Belgium 199; Luxembourg: 15.

15 From this population, a total of 144 samples were sequenced which allowed to separate the genotype B samples (94) from the non-B samples (50). After analysis of these genotyped samples on LiPA, the genotypic reactivity on the selected probes was scored. Figures 4A to 4F show these results for the different codon positions and for the genotype B versus non-B group. From these tables, it is clear that there is little difference in sequence usage for the different codon positions with respect to specific reactivities at the different probes.

20 The total collection of 856 samples was then tested on the available 46 probes. After dissection of these reactivities over the different probes and different geographical origin, the picture looks as is presented in Figures 5A to 5F. Again here, the majority of the sequences used at the different codon positions are restricted to some very abundant wild type motifs. It is important to mention here that the majority of these samples are taken from patients never treated with protease inhibitors, en therefore, the 25 majority of the reactivities are found in wild type motifs. Nevertheless, it is clear from some codon positions that the variability at some codon positions in the mutant motif might be considerable, and again, a continuos update on heavily treated patients is mandatory. Another issue is the amount of double blank reactivities, which is in this approach reaching up to 5% in global; with some peak values for some countries for some codon positions: for example 13.8% for codon 82/85 in Brazil; and 18.1 % for codon 30 90 in Belgium.

The continuous update resulted in a further selection of probes. This later configuration of the strip is indicated in table 7.

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Table 1

|          | 26  | 27  | 28  | 29  | 30  | 31  | 32  | 33  | 34  | 3   | Tm | length | Seq | ID  |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|----|--------|-----|-----|
|          | ACA | GGA | GCA | GAT | GAT | ACA | GTA | TTA | GAA | GAA |    |        |     |     |
| pc30w25  |     |     | GCA | GAT | GAT | ACA | GT  |     |     |     | 40 | 14     |     | 31  |
| pc30w29  |     |     | A   | GCG | GAT | GAT | ACA |     |     |     | 36 | 13     |     | 35  |
| pc30w32  |     |     |     | GCA | GAT | GAC | ACA | GT  |     |     | 42 | 14     |     | 38  |
| pc30w36  |     |     |     | GCA | GAC | GAT | ACA | GG  |     |     | 40 | 14     |     | 42  |
| pc30m23  |     |     | A   | GCA | GAT | AAT | ACA | GT  |     |     | 40 | 15     |     | 29  |
|          | 44  | 45  | 46  | 47  | 48  | 49  | 50  | 51  | 52  |     |    |        |     |     |
|          | CCA | AAA | ATG | ATA | GGG | GGA | ATT | GGA | GGT |     |    |        |     |     |
| pc48w47  |     |     |     | AAA | ATG | ATA | GGG | GGA |     |     | 42 | 15     |     | 93  |
| pc48w45  |     |     |     | A   | ATG | ATA | GGA | GGA | ATT |     | 42 | 16     |     | 91  |
| pc48w72  |     |     | A   | AAA | ATA | ATA | GGG | GGA |     |     | 42 | 16     |     | 120 |
| pc48m41  |     |     |     |     | ATG | ATA | GTG | GGA | ATT |     | 40 | 15     |     | 87  |
|          | 48  | 49  | 50  | 51  | 52  | 53  | 54  |     |     |     |    |        |     |     |
|          | GGG | GGA | ATT | GGA | GGT | TTT | ATC |     |     |     |    |        |     |     |
| pc50w31  |     |     |     | GGA | ATT | GGA | GGT | TTT |     |     | 42 | 15     |     | 151 |
| pc50w44  |     |     |     | GGA | ATT | GGG | GGT | TTG |     |     | 42 | 15     |     | 164 |
| pc50w52  |     |     |     | GA  | ATT | GGA | GTC | TTG |     |     |    | 14     |     | 172 |
| pc50m37  |     |     | GGG | GGA | GTT | GGA |     |     |     |     | 40 | 12     |     | 157 |
|          | 51  | 52  | 53  | 54  | 55  | 56  | 57  | 58  |     |     |    |        |     |     |
|          | GGA | GGT | TTT | ATC | AAA | GTA | AGA | CAG |     |     |    |        |     |     |
| pc54w3   |     |     |     | GT  | TTT | ATC | AAA | GTA | AGA |     | 42 | 17     |     | 178 |
| pc54w34  |     |     | GA  | GGT | TTT | ATC | AAA | GT  |     |     | 42 | 16     |     | 212 |
| pc54w14  |     |     |     | GGT | TTT | ATC | AAG | GTA | A   |     | 42 | 16     |     | 189 |
| pc54w19  |     |     | A   | GGC | TTT | ATC | AAA | GTA |     |     | 42 | 16     |     | 194 |
| pc54w22  |     |     | GA  | GGT | TTT | ATT | AAA | GTA |     |     | 42 | 17     |     | 197 |
| pc54w26  |     |     | A   | GGT | TTC | ATT | AAG | GTA |     |     | 42 | 16     |     | 202 |
| pc54w27  |     |     |     | GGT | TTT | ATT | AAG | GTA | A   |     | 40 | 16     |     | 204 |
| pc54m55  |     |     | A   | GGT | TTT | GCC | AAA | GT  |     |     | 38 | 15     |     |     |
| pc54m35  |     |     |     | GGT | TTT | GTC | AAA | GTA |     |     | 40 | 15     |     | 213 |
| pc54m37  |     |     | GGT | TTT | GTC | AGA | GTA |     |     |     | 42 | 15     |     | 215 |
|          | 78  | 79  | 80  | 81  | 82  | 83  | 84  | 85  | 86  | 87  |    |        |     |     |
|          | GGA | CCT | ACA | CCT | GTC | AAC | ATA | ATT | GGA | AGA |    |        |     |     |
| pc82w91  |     |     |     | ACA | CCT | GTC | AAC | ATA | A   |     | 44 | 16     |     | 318 |
| pc82w60  |     |     |     | CA  | CCT | GTC | AAT | ATA | ATG |     | 42 | 17     |     | 287 |
| pc82w111 |     |     |     | A   | CCG | GTC | AAC | ATA | ATT |     | 44 | 16     |     | 338 |
| pc82w89  |     |     | ACA | CCT | GTT | AAC | ATA | AG  |     |     | 42 | 17     |     | 316 |
| pc82w42  |     |     | CA  | CCT | GTC | AAC | GTA |     |     |     | 42 | 14     |     | 269 |
| pc82m36  |     |     | ACA | CCT | ACC | AAC | ATA |     |     |     | 42 | 15     |     | 263 |
| pc82m67  |     |     | ACA | CCT | ACC | AAC | GT  |     |     |     | 42 | 14     |     | 294 |
| pc82m38  |     |     | ACA | CCT | TTC | AAC | ATA |     |     |     | 40 | 15     |     | 265 |
| pc82m105 |     |     | ACG | CCC | TTC | AAC | ATA |     |     |     | 44 | 15     |     | 332 |
| pc82m127 |     |     | CA  | CCT | TTC | AAC | GTA | ATG |     |     | 44 | 17     |     | 354 |
| pc82m40  |     |     | ACA | CCT | GCC | AAC | ATA |     |     |     | 44 | 15     |     | 267 |
| pc82m63  |     |     | CA  | CCT | GCC | AAT | ATA | AG  |     |     | 42 | 16     |     | 290 |
| pc82m101 |     |     | ACA | CCT | ATC | AAC | ATA | ATG |     |     | 44 | 18     |     | 328 |

Table 1 - Cont'd

|         | 86  | 87  | 88  | 89  | 90  | 91  | 92  | 93  | 94    |    |    |     |
|---------|-----|-----|-----|-----|-----|-----|-----|-----|-------|----|----|-----|
|         | GGA | AGA | AAT | CTG | TTG | ACT | CAG | ATT | GGT   |    |    |     |
| pc90w27 |     |     | AAT | CTG | TTG | ACT | CA  |     |       | 38 | 14 | 384 |
| pc90w37 |     |     | AAT | CTG | TTG | ACT | CAG | ATG |       | 42 | 18 | 394 |
| pc90w39 |     |     | GA  | ACT | CTG | TTG | ACT | C   |       | 44 | 15 | 396 |
| pc90w50 |     |     | AAT | ATG | TTG | ACT | CAG |     |       | 40 | 15 | 407 |
| pc90w52 |     |     | AAT | TTG | TTG | ACT | CAG |     |       | 40 | 15 | 409 |
| pc90w69 |     |     | GA  | AAC | CTG | TTG | ACT |     |       | 40 | 14 | 426 |
| pc90w73 |     |     |     |     | TG  | TTG | ACA | CAG | CTT G | 44 | 15 | 430 |
| pc90w79 |     |     |     |     | TG  | TTG | ACC | CAG | ATT G | 44 | 15 | 436 |
| pc90m43 |     | A   | AAT | CTG | ATG | ACT | CA  |     |       | 40 | 15 | 400 |
| pc90m56 |     |     | AAT | ATG | ATG | ACC | CAG |     |       | 42 | 15 | 413 |

**Table 2**  
**Protease Inhibitors**

| Compound                   | Amino acid change | Codon change |
|----------------------------|-------------------|--------------|
| <b>Protease Inhibitors</b> |                   |              |
| A-77003                    | R8Q               | CGA to CAA   |
|                            | R8K               | CGA to AAA   |
|                            | V32I              | GTA to ATA   |
|                            | M46I              | ATG to ATA   |
|                            | M46L              | ATG to TTC   |
|                            | M46F              | ATG to TTC   |
|                            | M46V              | ATG to GTG   |
|                            | G48V              | GGG to GTG   |
|                            | A71V              | GCT to GTT   |
|                            | V82I              | GTC to ATC   |
|                            | V82A              | GTC to GCC   |
|                            | L63P              | CTC to CCC   |
|                            | A71T              | GCT to ACT   |
|                            | A71V              | GCT to GTT   |
|                            | G73S              | GGT to GCT   |
|                            | V82A              | GTC to GCC   |
|                            | V82F              | GTC to TTC   |
|                            | V82T              | GTC to ACC   |
|                            | I84V              | ATA to GTA   |
|                            | L90M              | TTG to ATG   |
| P9941                      | V82A              | GTC to GCC   |
| Ro 31-8959<br>(saquinavir) | L10I              | CTC to ATC   |
|                            | G48V              | GGG to GTG   |
|                            | I54V              | ATC to GTC   |
|                            | I54V              | ATA to GTA   |
|                            | G73S              | GGT to AGT   |
|                            | V82A              | GTC to GCC   |
|                            | I84V              | ATA to GTA   |
|                            | L90M              | TTG to ATG   |
| RPI-312                    | I84V              | ATA to GTA   |

29  
Table 2 - Cont'd-1

|                                   |  |  |
|-----------------------------------|--|--|
| SC-52151                          | L24V<br>G48V<br>A71V<br>V75I<br>P81T<br>V82A<br>N88D     | TTA to GTA<br>GGG to GTG<br>GCT to GTT<br>GTA to ATA<br>CCT to ACT<br>GTC to GCC<br>AAT to GAT     |
| SC-55389A                         | L10F<br>N88S   | CTC to CGC<br>AAT to AGT   |
| SKF108842                         | V82T<br>I84V   | GTC to ACC<br>ATA to GTA   |
| SKF108922                         | V82A<br>V82T   | GTC to GCC<br>GTC to ACC   |
| VB 11,328                         | L10F<br>M46I<br>I47V<br>I50V<br>I84V                     | CTC to GGC<br>ATG to ATA<br>ATA to CTA<br>ATT to GTT<br>ATA to GTA                                 |
| VX-478<br>(141W94)                | L10F<br>M46I<br>I47V<br>I50V<br>I84V                     | CTC to CGC<br>ATG to ATA<br>ATA to CTA<br>ATT to GTT<br>ATA to GTA                                 |
| XM323                             | L10F<br>K45I<br>M46L<br>V82A<br><br>V82I<br>V82F<br>I84V | CTC to CGC<br>AAA to ATA<br>ATG to CTG<br>GTC to GCC<br><br>GTC to ATC<br>GTC to TTC<br>ATA to GTA |
|                                   | L97V   | TTA to GTA   |
|                                   | I82T   | ATC to ACC   |
| A-75925<br>ABT-538<br>(ritonavir) | V32I<br>K20R<br>L33F                                     | GTA to ATA<br>AAG to AAA<br>TTA to TTC   |

Table 2 - Cont'd-2

|                          |  |  |
|--------------------------|--|--|
|                          | M36I<br>M46I<br>I54L<br>I54V<br>A71V<br>V82F<br>V82A<br>V82T<br>V82S<br>I84V<br>L90M | ATG to ATA<br>ATG to ATA<br>ATC to ?<br>ATC to GTC<br>GTC to GTT<br>GTC to TTC<br>GTC to GCC<br>GTC to ACC<br>GTC to TCC<br>ATA to GTA<br>TTG to ATG |
| AG1343<br>(nelfinavir)   | D30N<br>M36I<br>M46I<br>L63P<br>A71V<br>V77I<br>I84V<br>N88D<br>L90M                 | GAT to AAT<br><br>ATG to ATA<br>CTC to CCC<br>GCT to GTT<br><br>ATA to GTA<br><br>TTG to ATG   |
| BILA 1906<br>BS          | V32I<br>M46I<br>M46L<br><br>A71V<br>I84A<br>I84V                                     | GTA to ATA<br>ATG to ATA<br>ATG to TTG<br><br>GCT to GTT<br>ATA to GCA<br>ATA to GTA   |
| BILA 2011<br>(palinavir) | V32I<br>A71V<br>I84A<br>L63P   | GTA to ATA<br>GCT to GTT<br>ATG to ATA<br>CTC to CCC   |
| BILA 2185 BS             | L23I   | CTA to ATA   |
| BMS 186,318              | A71T<br>V82A   | GCT to ACT<br>GTC to GCC   |
| DMP 450                  | L10F   | CTC to TTC   |

Table 2 - Cont'd-3

|                                     |  |  |
|-------------------------------------|--|--|
| M46I                                | ATG to ATA   |  |
| D60E                                | GAT to GAA   |  |
| I84V                                | ATA to GTA   |  |
| <br>                                | <br>   |  |
| KNI-272                             | V32I   |  |
| <br>                                | <br>   |  |
| MK-639<br>(L-735,524,<br>indinavir) | L10I<br>L10R<br>L10V<br>K20M<br>K20R<br>L24I<br>V32I<br>M46I<br>M46L<br>I54V | CTC to ATC<br>CTC to CGC<br>CTC to GTC<br>AAG to ATG<br>AAG to AAA<br>TTA to ATA<br>GTA to ATA<br>ATG to ATA<br>ATG to TTG<br>ATC to GTC |

Table 3

|        | 26  | 27  | 28  | 29  | 30  | 31  | 32  | 33  | 34  | 35  | length | Seq | ID |
|--------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--------|-----|----|
|        | ACA | GGA | GCA | GAT | GAT | ACA | GTA | TTA | GAA | GAA |        |     |    |
| P30w1  |     | A   | GCA | GAT | GAT | ACA | GTA | TT  |     |     | 18     |     | 7  |
| P30w2  |     | GA  | GCA | GAT | GAT | ACA | GTA | TT  |     |     | 19     |     | 8  |
| P30w3  |     | A   | GCA | GAT | GAT | ACA | GTA | TTA |     |     | 19     |     | 9  |
| P30w4  |     | GGA | GCA | GAT | GAT | ACA | GTA | TT  |     |     | 20     |     | 10 |
| P30w5  |     | GGA | GCA | GAT | GAT | ACA | GTA | TTA |     |     | 21     |     | 11 |
| P30w6  | ACA | GGA | GCA | GAT | GAT | ACA |     |     |     |     |        | 18  | 12 |
| P30w7  | CA  | GGA | GCA | GAT | GAT | ACA | GT  |     |     |     |        | 19  | 13 |
| P30w8  | A   | GGA | GCA | GAT | GAT | ACA | GTA | TG  |     |     | 20     |     | 14 |
| P30w9  |     | GGA | GCA | GAT | GAT | ACA | GTA | TG  |     |     | 19     |     | 15 |
| P30w10 | ACA | GGA | GCA | GAT | GAT | ACA | GG  |     |     |     | 19     |     | 16 |
| P30m11 |     | A   | GCA | GAT | AAT | ACA | GTA | TT  |     |     | 18     |     | 17 |
| P30m12 |     | GA  | GCA | GAT | AAT | ACA | GTA | TT  |     |     | 19     |     | 18 |
| P30m13 |     | A   | GCA | GAT | AAT | ACA | GTA | TTA |     |     | 19     |     | 19 |
| P30m14 |     | GGA | GCA | GAT | AAT | ACA | GTA | TT  |     |     | 20     |     | 20 |
| P30m15 |     | GGA | GCA | GAT | AAT | ACA | GTA | TTA |     |     | 21     |     | 21 |
| P30m15 | ACA | GGA | GCA | GAT | AAT | ACA |     |     |     |     | 18     |     | 22 |
| P30m17 | CA  | GGA | GCA | GAT | AAT | ACA | GT  |     |     |     | 19     |     | 23 |
| P30m18 | A   | GGA | GCA | GAT | AAT | ACA | GTA | TG  |     |     | 20     |     | 24 |
| P30m19 |     | GGA | GCA | GAT | AAT | ACA | GTA | TG  |     |     | 19     |     | 25 |
| P30m20 | ACA | GGA | GCA | GAT | AAT | ACA | GG  |     |     |     | 19     |     | 26 |
| p30w21 |     | A   | GCA | GAT | GAT | ACA | GT  |     |     |     | 15     |     | 27 |
| p30w22 |     | A   | GCA | GAT | GAT | ACA | GTA | G   |     |     | 16     |     | 28 |
| p30m23 |     | A   | GCA | GAT | AAT | ACA | GTA |     |     |     | 15     |     | 29 |
| p30m24 |     | A   | GCA | GAT | AAT | ACA | GTA | G   |     |     | 16     |     | 30 |
| p30w25 |     |     | GCA | GAT | GAT | ACA | GT  |     |     |     | 14     |     | 31 |
| p30w26 |     | A   | GCA | GAT | GAT | ACA | GG  |     |     |     | 14     |     | 32 |
| p30w27 |     |     | CA  | GAT | GAT | ACA | GT  |     |     |     | 13     |     | 33 |
| p30w28 |     | GA  | GCG | GAT | GAT | ACA |     |     |     |     | 14     |     | 34 |
| p30w29 |     | A   | GCG | GAT | GAT | ACA |     |     |     |     | 13     |     | 35 |
| p30m30 |     |     | GCA | GAT | AAT | ACA | GTA |     |     |     | 15     |     | 36 |
| p30m31 |     |     | GCA | GAT | AAT | ACA | GT  |     |     |     | 14     |     | 37 |
| p30w32 |     |     | GCA | GAT | GAC | ACA | GT  |     |     |     | 14     |     | 38 |
| p30w33 |     |     | CA  | GAT | GAC | ACA | GTA | G   |     |     | 14     |     | 39 |
| p30w34 |     |     | CA  | GAT | GAT | ACA | ATA | TT  |     |     | 16     |     | 40 |
| p30w35 |     |     | GCA | GAT | GAT | ACA | ATA | TG  |     |     | 16     |     | 41 |
| p30w36 |     |     | GCA | GAC | GAT | ACA | GG  |     |     |     | 13     |     | 42 |
| p30w37 |     |     | GCA | GAC | GAT | ACA | GT  |     |     |     | 14     |     | 43 |
| p30w38 |     |     | A   | GAT | GAT | ACA | ATA | TT  |     |     | 15     |     | 44 |
| p30w39 |     |     | A   | GAT | GAT | ACA | ATA | TTA |     |     | 16     |     | 45 |
| p30w40 |     |     | GCA | GAT | GAT | ACA | ATA |     |     |     | 15     |     | 46 |

Table 3 - Cont'd-1

|        | 44  | 45  | 46  | 47  | 48  | 49  | 50  | 51  | 52  | 53  | 54  | length | Seq | ID |
|--------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--------|-----|----|
|        | CCA | AAA | ATG | ATA | GGG | GGA | ATT | GGA | GGT | TTT | ATC |        |     |    |
| P48w1  |     |     |     |     | GTA | GGG | GGA | ATT | GGA | GGT | GG  | 18     | 47  |    |
| P48w2  |     |     |     |     | GTA | GGG | GGA | ATT | GGA | GGT | TG  | 19     | 48  |    |
| P48w3  |     |     |     |     | GTA | GGG | GGA | ATT | GGA | GGT | TTG | 20     | 49  |    |
| P48w4  |     |     |     |     | GTA | GGG | GGA | ATT | GGA | GGT | TTT | 21     | 50  |    |
| P48w5  |     |     |     | G   | GTA | GGG | GGA | ATT | GGA | GGT | TTG | 21     | 51  |    |
| P48w6  |     |     | ATG |     | GTA | GGG | GGA | ATT | GGA |     |     | 18     | 52  |    |
| P48w7  |     |     | ATG |     | GTA | GGG | GGA | ATT | GGA | G   |     | 19     | 53  |    |
| P48w8  |     | A   | ATG |     | GTA | GGG | GGA | ATT | GGA |     |     | 19     | 54  |    |
| P48w9  |     | A   | ATG |     | GTA | GGG | GGA | ATT | GGA | G   |     | 20     | 55  |    |
| P48w10 |     | A   | ATG |     | GTA | GGG | GGA | ATT | GGA | GGG | GG  | 22     | 56  |    |
| P48w21 |     |     | ATA | ATA | GGG | GGA | ATT | GGA |     |     |     | 18     | 57  |    |
| P48w22 |     |     | ATG | ATA | GGG | GGA | ATT | GGA |     |     |     | 18     | 58  |    |
| P48w23 |     | A   | ATA | ATA | GGG | GGA | ATT | GGA |     |     |     | 19     | 59  |    |
| P48w24 |     | A   | ATG | ATA | GGG | GGA | ATT | GGA |     |     |     | 19     | 60  |    |
| P48w25 |     |     |     |     | ATA | GGG | GGA | ATT | GGA | GGT | GG  | 18     | 61  |    |
| P48w26 |     |     |     |     | ATA | GGG | GGA | ATT | GGA | GGT | TG  | 19     | 62  |    |
| P48w28 |     |     |     |     | ATA | GGG | GGA | ATT | GGA | GGT | TTG | 20     | 63  |    |
| P48w29 |     |     |     |     | ATA | GGG | GGA | ATT | GGA | GGT | TTT | 21     | 64  |    |
| P48m11 |     |     |     |     | GTA | GTG | GGA | ATT | GGA | GGT | GG  | 18     | 65  |    |
| P48m12 |     |     |     |     | GTA | GTG | GGA | ATT | GGA | GGT | TG  | 19     | 66  |    |
| P48m13 |     |     |     |     | GTA | GTG | GGA | ATT | GGA | GGT | TTG | 20     | 67  |    |
| P48m14 |     |     |     |     | GTA | GTG | GGA | ATT | GGA | GGT | TTT | 21     | 68  |    |
| P48m15 |     |     |     | G   | GTA | GTG | GGA | ATT | GGA | GGT | TTG | 21     | 69  |    |
| P48m16 |     |     | ATG |     | GTA | GTG | GGA | ATT | GGA |     |     | 18     | 70  |    |
| P48m17 |     |     | ATG |     | GTA | GTG | GGA | ATT | GGA | G   |     | 19     | 71  |    |
| P48m18 |     | A   | ATG |     | GTA | GTG | GGA | ATT | GGA |     |     | 19     | 72  |    |
| P48m19 |     | A   | ATG |     | GTA | GTG | GGA | ATT | GGA | G   |     | 20     | 73  |    |
| P48m20 |     | A   | ATG |     | GTA | GTG | GGA | ATT | GGA | GGG | GG  | 22     | 74  |    |
| P48m29 |     |     |     |     | ATA | GTG | GGA | ATT | GGA | GGT | GG  | 18     | 75  |    |
| P48m30 |     |     |     |     | ATA | GTG | GGA | ATT | GGA | GGT | TG  | 19     | 76  |    |
| P48m31 |     |     | ATG | ATA | GTG | GGA | ATT | GGA |     |     |     | 18     | 77  |    |
| P48m32 |     |     | ATG | ATA | GTG | GGA | ATT | GGA | G   |     |     | 19     | 78  |    |
| P48m33 |     | A   | ATG | ATA | GTG | GGA | ATT | GGA |     |     |     | 19     | 79  |    |
| p48w34 |     |     |     | G   | ATA | GGG | GGA | ATT | G   |     |     | 14     | 80  |    |
| p48w35 |     |     |     | TG  | ATA | GGG | GGA | ATT | G   |     |     | 15     | 81  |    |
| p48w36 |     |     |     | TG  | ATA | GGG | GGA | ATT | GG  |     |     | 16     | 82  |    |
| p48w37 |     |     |     | ATG | ATA | GGG | GGA | ATT |     |     |     | 15     | 83  |    |
| p48m38 |     |     |     | G   | ATA | GTG | GGA | ATT | G   |     |     | 14     | 84  |    |
| p48m39 |     |     |     | TG  | ATA | GTG | GGA | ATT | G   |     |     | 15     | 85  |    |
| p48m40 |     |     |     | TG  | ATA | GTG | GGA | ATT | GG  |     |     | 16     | 86  |    |
| p48m41 |     |     |     | ATG | ATA | GTG | GGA | ATT |     |     |     | 15     | 87  |    |
| p48w42 |     |     |     | ATA | ATA | GGG | GGA | ATT |     |     |     | 15     | 88  |    |
| p48w43 |     |     |     | TG  | ATA | GGG | GGA | GTT |     |     |     | 14     | 89  |    |
| p48w44 |     |     |     | G   | ATA | GGG | GGA | GTT | G   |     |     | 14     | 90  |    |
| p48w45 |     |     | A   | ATG | ATA | GGA | GGA | ATT |     |     |     | 16     | 91  |    |
| p48w46 |     |     |     | ATG | ATA | GGG | GGA | ATT |     |     |     | 15     | 92  |    |
| p48w47 |     |     | AAA | ATG | ATA | GGG | GGA |     |     |     |     | 15     | 93  |    |
| p48w48 |     |     | A   | AAA | ATG | ATA | GGG | GG  |     |     |     | 15     | 94  |    |

Table 3 - Cont'd-2

|         |                        |    |     |
|---------|------------------------|----|-----|
| p48w49  | AA ATG ATA GGG GGA AG  | 15 | 95  |
| p48w50  | AAA ATA ATA GGG GGA AG | 16 | 96  |
| p48w51  | AAA ATA AAA AT         | 15 | 97  |
| p48m52  | AAA ATG ATA GTG GGA AG | 16 | 98  |
| p48w52b | AAA TTG ATA GGG GG     | 14 | 99  |
| p48m53  | AAA ATG ATA GTG GGA    | 15 | 100 |
| p48w53b | AAA TTG ATA GGG GGA    | 15 | 101 |
| p48w54  | CA AAA TTG ATA G       | 15 | 102 |
| p48w55  | ATG GTA GGG GGA ATT    | 15 | 103 |
| p48w56  | AA ATG GTA GGG GGA     | 14 | 104 |
| p48w57  | A AAA ATG GTA GGG G    | 14 | 105 |
| p48w58  | ATG ATA GGG GAA ATT    | 15 | 106 |
| p48w59  | ATA GGG GAA ATT GGA    | 15 | 107 |
| p48w60  | ATA GGG GAA ATT GGA G  | 16 | 108 |
| p48w61  | ATG ATA GGG GGG ATT    | 15 | 109 |
| p48w62  | ATA GGG GGG ATT GG     | 14 | 110 |
| p48w63  | A GGG GGG ATT GGA      | 13 | 111 |
| p48m64  | AAA ATA ATA GTG GGA    | 15 | 112 |
| p48m65  | A AAA ATA ATA GTG GGA  | 16 | 113 |
| p48m66  | CA AAA ATA ATA GTG GG  | 16 | 114 |
| p48m67  | AAA TTG ATA GTG GGA    | 15 | 115 |
| p48m68  | A AAA TTG ATA GTG GGA  | 16 | 116 |
| p48m69  | CA AAA TTG ATA GTG G   | 15 | 117 |
| p48w70  | AAA ATG ATA GGG GG     | 14 | 118 |
| p48w71  | A AAA ATG ATA GGG G    | 14 | 119 |
| pc48w72 | A AAA ATA ATA GGG GGA  | 16 | 120 |

Table 3 - Cont'd-3

|        | 45  | 46  | 47  | 48  | 49  | 50  | 51  | 52  | 53  | 54  | length | Seq | ID  |     |     |  |
|--------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--------|-----|-----|-----|-----|--|
|        | AAA | ATG | GTA | GGG | GGA | ATT | GGA | GGT | TTT | ATC |        |     |     |     |     |  |
| P50w1  |     |     |     | GGG | GGA | ATT | GGA | GGT | TTT |     | 18     | 121 |     |     |     |  |
| P50w2  |     |     |     | A   | GGG | GGA | ATT | GGA | GGT | TTT |        | 19  | 122 |     |     |  |
| P50w3  |     |     |     | TA  | GGG | GGA | ATT | GGA | GGT | TTT |        | 20  | 123 |     |     |  |
| P50w4  |     |     |     | A   | GGG | GGA | ATT | GGA | GGT | TTT | AG     | 20  | 124 |     |     |  |
| P50w5  |     |     |     | TA  | GGG | GGA | ATT | GGA | GGT | TTT | AG     | 21  | 125 |     |     |  |
| P50w6  |     |     |     | GTA | GGG | GGA | ATT | GGA | GGT | TGG |        | 19  | 126 |     |     |  |
| P50w7  |     |     |     | G   | GTA | GGG | GGA | ATT | GGA | GGT | TGG    |     | 20  | 127 |     |  |
| P50w8  |     |     |     | GTA | GGG | GGA | ATT | GGA | GGT | TTG |        | 20  | 128 |     |     |  |
| P50w9  |     |     |     | GTA | GGG | GGA | ATT | GGA | GGT | TTT |        | 20  | 129 |     |     |  |
| P50w10 |     |     |     | TG  | GTA | GGG | GGA | ATT | GGA | GGT | GG     |     | 20  | 130 |     |  |
| p50w21 |     |     |     |     | GG  | GGA | ATT | GGA | GGT | TTT |        | 17  | 131 |     |     |  |
| P50w22 |     |     |     |     | GG  | GGA | ATT | GGA | GGT | TTG |        | 16  | 132 |     |     |  |
| P50w23 |     |     |     |     | GG  | GGA | ATT | GGA | GGT | TTT | AG     | 18  | 133 |     |     |  |
| P50w24 |     |     |     |     | GG  | GGA | ATT | GGA | GGT | TG  |        | 15  | 134 |     |     |  |
| P50w25 |     |     |     |     | G   | GGA | ATT | GGA | GGT | TTT | AT     | 18  | 135 |     |     |  |
| P50w26 |     |     |     |     | GG  | GGA | ATT | GGA | GGT | TTT |        | 17  | 136 |     |     |  |
| P50m11 |     |     |     |     | GGG | GGA | GTT | GGA | GGT | TTT |        | 18  | 137 |     |     |  |
| P50m12 |     |     |     |     | A   | GGG | GGA | GTT | GGA | GGT | TTT    |     | 19  | 138 |     |  |
| P50m13 |     |     |     |     | TA  | GGG | GGA | GTT | GGA | GGT | TTT    |     | 20  | 139 |     |  |
| P50m14 |     |     |     |     | A   | GGG | GGA | GTT | GGA | GGT | TTT    | AG  | 20  | 140 |     |  |
| P50m15 |     |     |     |     | TA  | GGG | GGA | GTT | GGA | GGT | TTT    | AG  | 21  | 141 |     |  |
| P50m16 |     |     |     |     | GTA | GGG | GGA | GTT | GGA | GGT | TGG    |     | 19  | 142 |     |  |
| P50m17 |     |     |     |     | G   | GTA | GGG | GGA | GTT | GGA | GGT    | TGG |     | 20  | 143 |  |
| P50m18 |     |     |     |     | GTA | GGG | GGA | GTT | GGA | GGT | TTG    |     | 20  | 144 |     |  |
| P50m19 |     |     |     |     | GTA | GGG | GGA | GTT | GGA | GGT | TTT    | ATC | 21  | 145 |     |  |
| P50m20 |     |     |     |     | TG  | GTA | GGG | GGA | GTT | GGA | GGT    | GG  |     | 20  | 146 |  |
| P50m27 |     |     |     |     |     | GG  | GGA | GTT | GGA | GGT | TTG    |     | 19  | 147 |     |  |
| P50m28 |     |     |     |     |     | GG  | GGA | GTT | GGA | GGT | TTT    | AG  | 18  | 148 |     |  |
| P50m29 |     |     |     |     |     | GG  | GGA | GTT | GGA | GGT | TG     |     | 15  | 149 |     |  |
| P50m30 |     |     |     |     |     | G   | GGA | GTT | GGA | GGT | TTT    | AT  | 18  | 150 |     |  |
| p50w31 |     |     |     |     |     | GGA | ATT | GGA | GGT | TTT |        |     | 15  | 151 |     |  |
| p50w32 |     |     |     |     |     | G   | GGA | ATT | GGA | GGT | TGG    |     | 15  | 152 |     |  |
| p50m33 |     |     |     |     |     | GGA | GTT | GGA | GGT | TTT |        |     | 15  | 153 |     |  |
| p50m34 |     |     |     |     |     | G   | GGA | GTT | GGA | GGT | TGG    |     | 14  | 154 |     |  |
| p50m35 |     |     |     |     |     | GGG | GGA | GTT | GGA | G   |        |     | 13  | 155 |     |  |
| p50m36 |     |     |     |     |     | GG  | GGA | GTT | GGA | G   |        |     | 12  | 156 |     |  |
| p50m37 |     |     |     |     |     | GGG | GGA | GTT | GGA |     |        |     | 12  | 157 |     |  |
| p50w38 |     |     |     |     |     | GGA | ATT | GGG | GGT | TTG |        |     | 14  | 158 |     |  |
| p50w39 |     |     |     |     |     | GA  | ATT | GGG | GGT | TTT |        |     | 14  | 159 |     |  |

Table 3 - Cont'd-4

|        |                       |    |     |
|--------|-----------------------|----|-----|
| p50w40 | GA ATT GGG GGT TTT AG | 15 | 160 |
| p50w41 | GGA ATT GGG GGT TG    | 13 | 161 |
| p50w42 | GGA ATT GGG GGT G     | 12 | 162 |
| p50w43 | GA ATT GGG GGT TG     | 12 | 163 |
| p50w44 | GA ATT GGG GGT TTG    | 13 | 164 |
| p50w45 | GGG GGA ATT GCA G     | 13 | 165 |
| p50w46 | GGA ATT GCA GGT TG    | 14 | 166 |
| p50w47 | GGA ATT GCA GGT G     | 13 | 167 |
| p50w48 | GGA ATT GGA GGG TTG   | 14 | 168 |
| p50w49 | GA ATT GGA GGG TTG    | 13 | 169 |
| p50w50 | GA ATT GGA GGG TTT    | 14 | 170 |
| p50w51 | GGA ATT GGA GGC TTG   | 14 | 171 |
| p50w52 | GA ATT GGA GGC TTG    | 13 | 172 |
| p50w53 | GA ATT GGA GGC TTT    | 14 | 173 |
| p50m54 | GGA GTT GGA GGT TTG   | 15 | 174 |
| p50m55 | GA GTT GGA GGT TTT    | 14 | 175 |

Table 3 - Cont'd-5

|        | 51  | 52  | 53  | 54  | 55  | 56  | 57  | 58  | length | Seq | ID  |  |
|--------|-----|-----|-----|-----|-----|-----|-----|-----|--------|-----|-----|--|
|        | GGA | GGT | TTT | ATC | AAA | GTA | AGA | CAG |        |     |     |  |
| p54w1  |     | GGT | TTT | ATC | AAA | GTA | A   |     | 16     | 176 |     |  |
| p54w2  |     |     | GT  | TTT | ATC | AAA | GTA | AG  | 16     | 177 |     |  |
| p54w3  |     |     |     | GT  | TTT | ATC | AAA | GTA | AGA    | 17  | 178 |  |
| p54w4  |     |     |     | T   | TTT | ATC | AAA | GTA | AGA    | 16  | 179 |  |
| p54w5  |     | GGT | TTT | ATC | AAA | GTA |     |     | 15     | 180 |     |  |
| p54w6  |     |     |     | GT  | TTT | ATC | AAA | GTA |        | 15  | 181 |  |
| p54m7  |     | GGT | TTT | GCC | AAA | GTA |     |     | 15     | 182 |     |  |
| p54m8  |     |     |     | GT  | TTT | GCC | AAA | GTA | A      | 15  | 183 |  |
| p54m9  |     |     |     | GT  | TTT | GCC | AAA | GTA | AG     | 16  | 184 |  |
| p54m10 |     |     |     | T   | TTT | GCC | AAA | GTA | AGA    | 16  | 185 |  |
| p54m11 |     | GGT | TTT | GCC | AAA | GT  |     |     | 14     | 186 |     |  |
| p54m12 |     |     |     | GT  | TTT | GCC | AAA | GTA |        | 14  | 187 |  |
| p54w13 |     |     |     | GT  | TTT | ATC | AAG | GTA | AA     | 16  | 188 |  |
| p54w14 |     | GGT | TTT | ATC | AAG | GTA | A   |     | 16     | 189 |     |  |
| p54w15 | A   | GGT | TTT | ATC | AAG | GTA |     |     | 16     | 190 |     |  |
| p54w16 |     |     | GT  | TTT | ATC | AAA | GTC | AGA | 17     | 191 |     |  |
| p54w17 |     |     |     | TTT | ATC | AAA | GTC | AGA | C      | 16  | 192 |  |
| p54w18 | A   | GGC | TTT | ATC | AAA | GTA | A   |     | 17     | 193 |     |  |
| p54w19 | A   | GGC | TTT | ATC | AAA | GTA |     |     | 16     | 194 |     |  |
| p54m20 | A   | GGT | TTT | ATT | AAA | GTA | A   |     | 17     | 195 |     |  |
| p54m21 |     | GGT | TTT | ATT | AAA | GTA | AG  |     | 17     | 196 |     |  |
| p54w22 | GA  | GGT | TTT | ATT | AAA | GTA |     |     | 17     | 197 |     |  |
| p54m22 | GA  | GGT | TTT | ATT | AAA | GTA |     |     | 17     | 198 |     |  |
| p54m23 |     | GGT | TTT | ATT | GGT | TTT | AT  |     | 16     | 199 |     |  |
| p54m24 |     | GGT | TTC | ATT | AAG | GTA |     |     | 15     | 200 |     |  |
| p54m25 |     | GGT | TTC | ATT | AAG | GTA | A   |     | 16     | 201 |     |  |
| p54w26 | A   | GGT | TTC | ATT | AAG | GTA |     |     | 16     | 202 |     |  |
| p54m26 | A   | GGT | TTC | ATT | AAG | GTA |     |     | 16     | 203 |     |  |
| p54w27 |     | GGT | TTT | ATT | AAG | GTA | A   |     | 16     | 204 |     |  |
| p54m27 |     | GGT | TTT | ATT | AAG | GTA | A   |     | 16     | 205 |     |  |
| p54m28 | A   | GGT | TTT | ATT | AAG | GTA |     |     | 16     | 206 |     |  |
| p54m29 | GA  | GGT | TTT | ATT | AAG | GT  |     |     | 16     | 207 |     |  |
| p54m30 |     | GGT | TTT | ATT | AAG | GTA | AG  |     | 17     | 208 |     |  |
| p54w31 |     | GGT | TTT | ATC | AAA | GTA | A   |     | 16     | 209 |     |  |
| p54w32 | A   | GGT | TTT | ATC | AAA | GTA | A   |     | 17     | 210 |     |  |
| p54w33 | A   | GGT | TTT | ATC | AAA | GTA |     |     | 16     | 211 |     |  |
| p54w34 | GA  | GGT | TTT | ATC | AAA | GT  |     |     | 16     | 212 |     |  |
| p54m35 |     | GGT | TTT | GTC | AAA | GTA |     |     | 15     | 213 |     |  |
| p54m36 |     | GGT | TTT | GTC | AAA | GTA | A   |     | 16     | 214 |     |  |
| p54m37 |     | GGT | TTT | GTC | AGA | GTA |     |     | 15     | 215 |     |  |
| p54m38 |     | GGT | TTT | GTC | AGA | GTA | A   |     | 16     | 216 |     |  |
| p54w39 |     | GGG | TTT | ATC | AAA | GTA |     |     | 15     | 217 |     |  |
| p54w40 |     | GGG | TTT | ATC | AAA | GTA | A   |     | 16     | 218 |     |  |
| p54w41 |     | GGC | TTC | ATC | AAA | GT  |     |     | 14     | 219 |     |  |
| p54w42 | GA  | GGC | TTC | ATC | AAA |     |     |     | 14     | 220 |     |  |
| p54m48 |     | GGT | TTT | GTC | AAA | GT  |     |     | 14     | 221 |     |  |
| p54m49 |     | GT  | TTT | GTC | AGA | GTA |     |     | 14     | 222 |     |  |

**Table 3 - Cont'd-6**

|        |                       |    |     |
|--------|-----------------------|----|-----|
| p54m50 | GGT TTT GTC AGA GT    | 14 | 223 |
| p54w51 | A GGT TTA ATC AAA GTA | 16 | 224 |
| p54w52 | GA GGT TTA ATC AAA GT | 16 | 225 |
| p54m53 | GGT TTT ACC AAA GTA   | 15 | 226 |
| p54m54 | GGT TTT ACC AAA GT    | 14 | 227 |

Table 3 - Cont'd-7

|        | 78  | 79  | 80  | 81  | 82  | 83  | 84  | 85  | 86  | 87  | length | Seq | ID |
|--------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--------|-----|----|
|        | GGA | CCT | ACA | CCT | GTC | AAC | ATA | ATT | GGA | AGA |        |     |    |
| P82w1  |     | CCT | ACA | CCT | GTC | AAC | ATA | AG  |     |     | 19     | 228 |    |
| P82w2  |     | CCT | ACA | CCT | GTC | AAC | ATA | ATG |     |     | 20     | 229 |    |
| P82w3  |     | CCT | ACA | CCT | GTC | AAC | ATA | ATT |     |     | 21     | 230 |    |
| P82w4  |     | A   | CCT | ACA | CCT | GTC | AAC | ATA | AG  |     | 20     | 231 |    |
| P82w5  |     | A   | CCT | ACA | CCT | GTC | AAC | ATA | ATG |     | 21     | 232 |    |
| P82w6  |     | A   | CCT | ACA | CCT | GTC | AAC | ATA |     |     | 19     | 233 |    |
| P82w7  |     | GA  | CCT | ACA | CCT | GTC | AAC | ATA |     |     | 20     | 234 |    |
| P82w8  |     |     | CA  | CCT | GTC | AAC | ATA | ATT | GGA |     | 20     | 235 |    |
| P82w9  |     |     | A   | CCT | GTC | AAC | ATA | ATT | GGA | A   | 20     | 236 |    |
| P82w10 |     |     | ACA | CCT | GTC | AAC | ATA | ATT | GG  |     | 20     | 237 |    |
| P82w21 |     |     | A   | CCT | GTC | AAC | ATA | ATT | GGA |     | 19     | 238 |    |
| P82m11 |     | CCT | ACA | CCT | ACC | AAC | ATA | AG  |     |     | 19     | 239 |    |
| P82m12 |     | CCT | ACA | CCT | ACC | AAC | ATA | ATG |     |     | 20     | 240 |    |
| P82m13 |     | CCT | ACA | CCT | ACC | AAC | ATA | ATT |     |     | 21     | 241 |    |
| P82m14 |     | A   | CCT | ACA | CCT | ACC | AAC | ATA | AG  |     | 20     | 242 |    |
| P82m15 |     | A   | CCT | ACA | CCT | ACC | AAC | ATA | ATG |     | 21     | 243 |    |
| P82m16 |     | A   | CCT | ACA | CCT | ACC | AAC | ATA |     |     | 19     | 244 |    |
| P82m17 |     | GA  | CCT | ACA | CCT | ACC | AAC | ATA |     |     | 20     | 245 |    |
| P82m18 |     |     | CA  | CCT | ACC | AAC | ATA | ATT | GGA |     | 20     | 246 |    |
| P82m19 |     |     | A   | CCT | ACC | AAC | ATA | ATT | GGA | A   | 20     | 247 |    |
| P82m20 |     |     | ACA | CCT | ACC | AAC | ATA | ATT | G   |     | 19     | 248 |    |
| P82m22 |     | CCT | ACA | CCT | TTC | AAC | ATA | ATT |     |     | 21     | 249 |    |
| P82m23 |     | CCT | ACA | CCT | GCC | AAC | ATA | ATT |     |     | 21     | 250 |    |
| P82m24 |     | CCT | ACA | CCT | TCC | AAC | ATA | ATT |     |     | 21     | 251 |    |
| P82m25 |     |     | A   | CCT | TTC | AAC | ATA | ATT | GGA | A   | 20     | 252 |    |
| P82m26 |     |     | A   | CCT | GCC | AAC | ATA | ATT | GGA | A   | 20     | 253 |    |
| P82m27 |     |     | A   | CCT | TTC | AAC | ATA | ATT | GGA | A   | 20     | 254 |    |
| P82m28 |     |     | A   | CCT | ACC | AAC | ATA | ATT |     |     | 16     | 255 |    |
| P82m29 |     |     | A   | CCT | TTC | AAC | ATA | ATT | GGA |     | 19     | 256 |    |
| P82m30 |     |     | A   | CCT | GCC | AAC | ATA | ATT | GGA |     | 19     | 257 |    |
| P82m31 |     |     | A   | CCT | TCC | AAC | ATA | ATT | GGA |     | 19     | 258 |    |
| P82w32 |     | T   | ACA | CCT | GTC | AAC | AT  |     |     |     | 15     | 259 |    |
| P82w33 |     | T   | ACA | CCT | GTC | AAC | ATA |     |     |     | 16     | 260 |    |
| P82w34 |     |     | ACA | CCT | GTC | AAC | ATA |     |     |     | 15     | 261 |    |
| P82w35 |     |     | CA  | CCT | GTC | AAC | ATA |     |     |     | 14     | 262 |    |
| P82m36 |     |     | ACA | CCT | ACC | AAC | ATA |     |     |     | 15     | 263 |    |
| P82m37 |     |     | CA  | CCT | ACC | AAC | ATA |     |     |     | 14     | 264 |    |
| P82m38 |     |     | ACA | CCT | TTC | AAC | ATA |     |     |     | 15     | 265 |    |
| P82m39 |     |     | CA  | CCT | TTC | AAC | ATA |     |     |     | 14     | 266 |    |
| P82m40 |     |     | ACA | CCT | GCC | AAC | ATA |     |     |     | 15     | 267 |    |
| P82m41 |     |     | CA  | CCT | GCC | AAC | ATA |     |     |     | 14     | 268 |    |
| P82w42 |     |     | CA  | CCT | GTC | AAC | GTA |     |     |     | 14     | 269 |    |
| P82w43 |     |     | CA  | CCT | GTC | AAC | GT  |     |     |     | 13     | 270 |    |
| P82w44 |     | CCT | ACA | CCT | GTC | AAC |     |     |     |     | 15     | 271 |    |
| P82w45 |     | T   | ACG | CCT | GTC | AAC | AT  |     |     |     | 15     | 272 |    |
| P82w46 |     | CT  | ACG | CCT | GTC | AAC | AG  |     |     |     | 15     | 273 |    |
| P82m47 |     |     | ACA | CCT | TCC | AAC | ATA |     |     |     | 15     | 274 |    |

Table 3 - Cont'd-8

|        |                         |    |     |
|--------|-------------------------|----|-----|
| P82m48 | CA CCT TCC AAC ATA      | 14 | 275 |
| P82m49 | ACA CCT TCC AAC AT      | 14 | 276 |
| P82m50 | ACA CCT ATC AAC ATA     | 15 | 277 |
| P82m51 | CA CCT ATC AAC ATA AG   | 15 | 278 |
| P82m52 | CA CCT ATC AAC ATA ATG  | 16 | 279 |
| P82m53 | A CCT ATC AAC ATA ATG   | 15 | 280 |
| P82w54 | CCT GTC AAC ATA ATT     | 15 | 281 |
| P82w55 | CCT GTT AAC ATA ATT G   | 16 | 282 |
| P82w56 | A CCT GTT AAC ATA ATG   | 15 | 283 |
| P82w57 | CCG GTC AAC ATA ATT     | 15 | 284 |
| P82w58 | ACG CCT GTC AAC AT      | 14 | 285 |
| P82w59 | CCT GTC AAT ATA ATT     | 15 | 286 |
| P82w60 | CA CCT GTC AAT ATA ATG  | 16 | 287 |
| P82w61 | ACA CCT GTC AAT ATA AG  | 16 | 288 |
| P82m62 | CCT GCC AAT ATA ATT     | 15 | 289 |
| P82m63 | CA CCT GCC AAT ATA AG   | 15 | 290 |
| P82m64 | CCT ACC AAC GTA ATT     | 15 | 291 |
| P82m65 | CCT ACC AAC GTA ATG     | 14 | 292 |
| P82m66 | CA CCT ACC AAC GTA      | 14 | 293 |
| P82m67 | ACA CCT ACC AAC GT      | 14 | 294 |
| P82m68 | CCT TTC AAC GTA ATT     | 15 | 295 |
| P82m69 | CA CCT TTC AAC GTA AG   | 15 | 296 |
| P82m70 | ACA CCT TTC AAC GTA     | 15 | 297 |
| P82m71 | A CCT TTC AAC GTA ATG   | 15 | 298 |
| p82w72 | CT GTC AAT ATA ATT G    | 15 | 299 |
| p82w73 | CCT GTC AAT ATA ATT G   | 16 | 300 |
| p82w74 | A CCT GTC AAT ATA ATT   | 16 | 301 |
| p82w75 | CT GTC AAT ATA ATT GG   | 16 | 302 |
| p82w76 | CCT ACG CCT GTC AA      | 14 | 303 |
| p82w77 | CT ACG CCT GTC AAC      | 14 | 304 |
| p82w78 | A CCT ACG CCT GTC AA    | 15 | 305 |
| p82w79 | A CCT ACG CCT GTC A     | 14 | 306 |
| p82w80 | T ACA CCG GTC AAC A     | 14 | 307 |
| p82w81 | CT ACA CCG GTC AA       | 13 | 308 |
| p82w82 | CCT ACA CCG GTC A       | 13 | 309 |
| p82w83 | CA CCT GTC AAC ATA A    | 15 | 310 |
| p82w84 | A CCT GTC AAC ATA AT    | 15 | 311 |
| p82w85 | CT ACA CCT GTC AAC A    | 15 | 312 |
| p82w86 | ACA CCT GTC AAC AT      | 14 | 313 |
| p82w87 | A CCT GTT AAC ATA ATT G | 17 | 314 |
| p82w88 | CA CCT GTT AAC ATA AG   | 15 | 315 |
| p82w89 | ACA CCT GTT AAC ATA AG  | 16 | 316 |
| p82w90 | TCA CCT GTC AAC ATA     | 14 | 317 |
| p82w91 | ACA CCT GTC AAC ATA A   | 16 | 318 |
| p82w92 | CA CCT GTC AAC ATA AT   | 16 | 319 |
| p82w93 | CCT GTC AAC ATA ATT     | 15 | 320 |
| p82w94 | A CCT GTC AAC ATA ATT   | 16 | 321 |
| p82w95 | CCT GTC AAC ATA ATT G   | 16 | 322 |
| P82w96 | CCT ACA CCT GTC AA      | 14 | 323 |
| p82w97 | T GTC AAC ATA ATT GG    | 15 | 324 |
| p82w98 | T GTC AAC ATA ATT GGA   | 16 | 325 |

Table 3 - Cont'd-9

|         |                         |    |     |
|---------|-------------------------|----|-----|
| p82m99  | ACA CCT TTC AAC ATA A   | 16 | 326 |
| p82m100 | T ACA CCT TTC AAC ATA   | 16 | 327 |
| p82m101 | ACA CCT ATC AAC ATA ATG | 17 | 328 |
| P82m102 | ACA CCT ATC AAC ATA AG  | 16 | 329 |
| p82m103 | CA CCT GCC AAT ATA ATG  | 16 | 330 |
| p82m104 | ACA CCT GCC AAT ATA AG  | 16 | 331 |
| p82m105 | ACG CCC TTC AAC ATA     | 15 | 332 |
| p82m106 | CG CCC TTC AAC ATA AG   | 15 | 333 |
| p82m107 | T ACG CCC TTC AAC AT    | 15 | 334 |
| p82w108 | CT ACA CCG GTC AAC      | 14 | 335 |
| p82w109 | CCT ACA CCG GTC AA      | 14 | 336 |
| p82w110 | A CCG GTC AAC ATA ATG   | 15 | 337 |
| p82w111 | A CCG GTC AAC ATA ATT   | 16 | 338 |
| p82w112 | CT ACA CCA GTC AAC      | 14 | 339 |
| p82w113 | CT ACA CCA GTC AAC A    | 15 | 340 |
| p82w114 | ACA CCA GTC AAC ATA     | 15 | 341 |
| p82w115 | ACA CCA GTC AAC ATA AG  | 16 | 342 |
| p82w116 | T ACG CCT GTC AAC AT    | 15 | 343 |
| p82w117 | ACG CCT GTC AAC ATA     | 15 | 344 |
| p82w118 | T ACG CCT GTC AAC A     | 14 | 345 |
| p82m119 | CCT ACA CCT TTC AAC     | 15 | 346 |
| p82m120 | CT ACA CCT TTC AAC      | 14 | 347 |
| p82m121 | A CCT ACA CCT TTC AA    | 15 | 348 |
| p82w122 | ACG CCT GTC AAC ATA AGG | 16 | 349 |
| p82w123 | T ACG CCT GTC AAC ATA   | 16 | 350 |
| p82w124 | CG CCT GTC AAC ATA AGG  | 15 | 351 |
| p82m125 | T ACA CCT TTC AAC GTA   | 16 | 352 |
| p82m126 | ACA CCT TTC AAC GTA AGG | 16 | 353 |
| p82m127 | CA CCT TTC AAC GTA ATG  | 16 | 354 |
| p82m128 | A CCT TTC AAC GTA ATT   | 16 | 355 |
| p82o129 | C AAC GTA ATT GGA AGA   | 16 | 356 |
| p82o130 | C AAC GTA ATT GGA AG    | 15 | 357 |

Table 3 - Cont'd-10

|        | 86  | 87  | 88  | 89  | 90  | 91  | 92  | 93    | 94  | length | Seq | ID |
|--------|-----|-----|-----|-----|-----|-----|-----|-------|-----|--------|-----|----|
|        | GGA | AGA | AAT | CTG | TTG | ACT | CAG | ATT   | GGT |        |     |    |
| P90w1  |     | A   | AAT | CTG | TTG | ACT | CAG |       |     | 16     | 358 |    |
| P90w2  |     | GA  | AAT | CTG | TTG | ACT | CAG |       |     | 17     | 359 |    |
| P90w3  |     | GA  | AAT | CTG | TTG | ACT | CAG | AGG   |     | 18     | 360 |    |
| P90w4  |     | A   | AAT | CTG | TTG | ACT | CAG | AGG   |     | 17     | 361 |    |
| P90w5  |     | AGA | AAT | CTG | TTG | ACT | CAG | AGG   |     | 19     | 362 |    |
| P90w6  |     | AGA | AAT | CTG | TTG | ACT | CAG | ATG   |     | 20     | 363 |    |
| P90w7  |     | AGA | AAT | CTG | TTG | ACT | CAG | ATT   |     | 21     | 364 |    |
| P90w8  |     | AGA | AAT | CTG | TTG | ACT | CAG | ATTGG |     | 20     | 365 |    |
| P90w9  |     | GA  | AGA | AAT | CTG | TTG | ACT | CAG   | AGG | 21     | 366 |    |
| P90w10 |     | A   | AGA | AAT | CTG | TTG | ACT | CAG   | ATG | 21     | 367 |    |
| P90m11 |     | AGA | AAT | CTG | ATG | ACT | CAG | ATG   |     | 20     | 368 |    |
| P90m12 |     | AGA | AAT | CTG | ATG | ACT | CAG | ATT   |     | 21     | 369 |    |
| P90m13 |     | A   | AGA | AAT | CTG | ATG | ACT | CAG   | AGG | 20     | 370 |    |
| P90m14 |     | GA  | AGA | AAT | CTG | ATG | ACT | CAG   | AGG | 21     | 371 |    |
| P90m15 |     | A   | AGA | AAT | CTG | ATG | ACT | CAG   | ATG | 21     | 372 |    |
| P90m16 |     | GA  | AGA | AAT | CTG | ATG | ACT | CAG   | ATT | 20     | 373 |    |
| P90m17 |     | GGA | AGA | AAT | CTG | ATG | ACT | CAG   |     | 21     | 374 |    |
| P90m18 |     | A   | AGA | AAT | CTG | ATG | ACT | CAG   |     | 19     | 375 |    |
| P90m19 |     | A   | AAT | CTG | ATG | ACT | CAG | ATT   | GG  | 21     | 376 |    |
| P90m20 |     | A   | AAT | CTG | ATG | ACT | CAG | ATT   | G   | 20     | 377 |    |
| P90m21 |     | A   | AAT | CTG | ATG | ACT | CAG | CTT   | G   | 20     | 378 |    |
| P90m22 |     | A   | AAT | CTG | ATG | ACT | CAG | CTT   |     | 19     | 379 |    |
| P90m23 |     |     | AAT | CTG | ATG | ACT | CAG | CTT   | G   | 18     | 380 |    |
| P90w24 |     | A   | AAT | CTG | TTG | ACT | CAG | CTT   | G   | 20     | 381 |    |
| P90w25 |     | A   | AAT | CTG | TTG | ACT | CAG | CTT   |     | 19     | 382 |    |
| P90w26 |     |     | AAT | CTG | TTG | ACT | CAG | CTT   | G   | 19     | 383 |    |
| P90w27 |     |     | AAT | CTG | TTG | ACT | CA  |       |     | 14     | 384 |    |
| P90w28 |     |     | AAT | CTG | TTG | ACT | CAG |       |     | 15     | 385 |    |
| P90w29 |     | A   | AAT | CTG | TTG | ACT | CA  |       |     | 15     | 386 |    |
| P90w30 |     | A   | AAT | CTG | TTG | ACT | CAG |       |     | 16     | 387 |    |
| P90m31 |     |     | AAT | CTG | ATG | ACT | CA  |       |     | 14     | 388 |    |
| P90m32 |     |     | AAT | CTG | ATG | ACT | CAG |       |     | 15     | 389 |    |
| P90m33 |     | A   | AAT | CTG | ATG | ACT | CA  |       |     | 15     | 390 |    |
| P90m34 |     | A   | AAT | CTG | ATG | ACT | CAG |       |     | 16     | 391 |    |
| P90w35 |     | GA  | AAT | CTG | TTG | ACT | C   |       |     | 15     | 392 |    |
| P90w36 |     | GA  | ACT | CTG | TTG | ACT | C   |       |     | 15     | 393 |    |
| P90w37 |     |     | T   | CTG | TTG | ACT | CAG | ATG   |     | 15     | 394 |    |
| P90w38 |     | GA  | AAT | CTG | TTG | ACT | C   |       |     | 15     | 395 |    |
| P90w39 |     | GA  | ACT | CTG | TTG | ACT | C   |       |     | 15     | 396 |    |
| P90w40 |     | A   | AAT | CTG | TTG | ACT | CA  |       |     | 15     | 397 |    |
| P90w41 |     |     | AAT | CTG | TTG | ACT | CAG |       |     | 15     | 398 |    |
| P90m42 |     |     | AAT | CTG | ATG | ACT | CAG |       |     | 15     | 399 |    |
| P90m43 |     | A   | AAT | CTG | ATG | ACT | CA  |       |     | 15     | 400 |    |
| P90w44 |     |     | AT  | CTG | TTG | ACT | CAG | AG    |     | 15     | 401 |    |
| P90w45 |     |     |     | CTG | TTG | ACT | CAG | ATT   |     | 15     | 402 |    |
| P90w46 |     | AGA | AAT | CTG | TTG | ACT |     |       |     | 15     | 403 |    |
| P90m47 |     |     | AT  | CTG | ATG | ACT | CAG | AG    |     | 15     | 404 |    |

Table 3 - Cont'd-11

|         |                        |    |     |
|---------|------------------------|----|-----|
| P90m48  | CTG ATG ACT CAG ATT    | 15 | 405 |
| P90m49  | AGA AAT CTG ATG ACT CA | 17 | 406 |
| P90w50  | AAT ATG TTG ACT CAG    | 15 | 407 |
| P90w51  | GA AAT ATG TTG ACT CA  | 16 | 408 |
| P90w52  | AAT TTG TTG ACT CAG    | 15 | 409 |
| P90w53  | GA AAT TTG TTG ACT CA  | 16 | 410 |
| P90w54  | AAT ATG TTG ACC CAG    | 15 | 411 |
| P90w55  | A AAT ATG TTG ACC CA   | 15 | 412 |
| P90m56  | AAT ATG ATG ACC CAG    | 15 | 413 |
| P90m57  | A CAG ATG ATG ACC CA   | 15 | 414 |
| P90w58  | AAC ATG TTG ACT CAG    | 15 | 415 |
| P90w59  | A AAC ATG TTG ACT CAG  | 15 | 416 |
| P90w60  | TG TTG ACT CAG CTT     | 14 | 417 |
| P90w61  | CTG TTG ACT CAG CTG    | 14 | 418 |
| P90m62  | CT ATG ACT CAG CTT     | 14 | 419 |
| P90m63  | CTG ATG ACT CAG C-G    | 14 | 420 |
| P90w64  | TG ACT ACA CAG CTT     | 14 | 421 |
| P90w65  | CTG TTG ACA CAG C-G    | 14 | 422 |
| P90w66  | AAT CTG TTG ACA CAG    | 15 | 423 |
| P90w67  | AAC CTG TTG ACT CA     | 13 | 424 |
| P90w68  | A AAC CTG TTG ACT C    | 13 | 425 |
| P90w69  | GA AAC CTG TTG ACT     | 13 | 426 |
| p90w70  | TG TTG ACT CAG ATT G   | 15 | 427 |
| p90w71  | TG TTG ACT CAG ATT GGG | 16 | 428 |
| p90w72  | G TTG ACT CAG ATT GGG  | 15 | 429 |
| p90w73  | TG TTG ACA CAG CTT G   | 15 | 430 |
| p90w74  | CTG TTG ACA CAG CTT    | 15 | 431 |
| p90w75  | G TTG ACA CAG CTT GGG  | 15 | 432 |
| p90w76  | TG TTG ACT CAG CTT G   | 15 | 433 |
| p90w77  | G TTG ACT CAG ATG      | 15 | 434 |
| p90w78  | G TTG ACT CAG CTT G    | 14 | 435 |
| p90w79  | TG TTG ACC CAG ATT G   | 15 | 436 |
| p90w80  | G TTG ACC CAG ATT G    | 14 | 437 |
| p90w81  | G TTG ACC CAG ATT GGG  | 15 | 438 |
| p90m82  | TG ATG ACT CAG ATT G   | 15 | 439 |
| p90m83  | TG ATG ACT CAG ATT GGG | 16 | 440 |
| p90m84  | G ATG ACT CAG ATT GGG  | 15 | 441 |
| p90m85  | G ATG ACT CAG ATT GGT  | 16 | 442 |
| p90m86  | CTG ATG ACT CAG CTT    | 15 | 443 |
| p90m87  | TG ATG ACT CAG CTT G   | 15 | 444 |
| P90w88  | A AAT CTG TTG ACT CA   | 15 | 445 |
| P90w89  | A AAT CTG TTG ACT CA   | 15 | 446 |
| p90w90  | A AAT CTG TTG ACT CA   | 15 | 447 |
| p90w100 | AAT CTG ATG ACT CAG    | 15 | 448 |
| p90m92  | A AAT CTG ATG ACT CA   | 16 | 449 |
| p90m93  | GA AAT CTG ATG ACT C   | 15 | 450 |
| p90m94  | CTG ATG ACT CAG ATG    | 15 | 451 |
| p90m95  | AGA AAT ATG ATG        | 15 | 452 |
| p90m96  | A AGA AAT ATG ATG ACT  | 16 | 453 |

Table 3 - Cont'd-12

|         |                           |    |     |
|---------|---------------------------|----|-----|
| p90m97  | A AGA AAT CTG ATG ACT     | 16 | 454 |
| p90m98  | A AGA AAT ATA ATG ACT     | 16 | 455 |
| p90m99  | A AAT ATA ATG ACT CAG     | 16 | 456 |
| p90m100 | AAT ATG ATG ACC CAG       | 15 | 457 |
| p90m101 | AAC CTG ATG ACT CAG       | 15 | 458 |
| p90m102 | AGA AAT TTG ATG ACT C     | 16 | 459 |
| p90m103 | A AAT TTG ATG ACT ATG ACT | 16 | 460 |
| p90m104 | AC CTG ATG ACT CAG        | 14 | 461 |
| p90m105 | AAT CTG ATG ACT CAG A     | 16 | 462 |
| p90m106 | AT CTG ATG ACT CAG ATG    | 16 | 463 |
| p90m107 | AT CTG ATG ACT CAG        | 14 | 464 |
| p90m108 | CTG ATG ACT CAG ATT G     | 16 | 465 |
| p90m109 | AGA AAT CTG ATG ACT C     | 16 | 466 |
| p90m110 | AGA AAT CTG ATG ACT       | 15 | 467 |
| p90m111 | GA AGA AAT CTG ATG A      | 15 | 468 |
| p90m112 | GGA AGA AAT CTG ATG A     | 16 | 469 |
| p90m113 | GA AGA AAT CTG ATG AC     | 16 | 470 |
| p90m114 | AGA AAT CTG ATG AC        | 14 | 471 |
| p90w115 | AAT CTG TTA ACT CAG       | 15 | 472 |
| p90w116 | T CTG TTA ACT CAG ATT     | 16 | 473 |
| p90w117 | AT CTG TTA ACT CAG AG     | 15 | 474 |
| p90w118 | AGA AAT TTG TTG ACT       | 16 | 475 |
| p90w119 | GA AAT TTG TTG ACT C      | 15 | 476 |
| p90w120 | AAT TTG TTG ACT CAG       | 15 | 477 |

Table 5

| probes for codon p30 |        | probes for codon p48 |       | probes for codon p50 |       | probes for codon p50 |       |
|----------------------|--------|----------------------|-------|----------------------|-------|----------------------|-------|
|                      | Type B | Type B               | non-B | Type B               | non-B | Type B               | non-B |
| w25                  | 95.7   | 98                   | w47   | 71.3                 | 70    | w31                  | 95.7  |
| w29                  | 1.1    | 0                    | w45   | 11.7                 | 22    | w44                  | 1.1   |
| w32                  | 1.1    | 1                    | w72   | 16                   | 4     | w52                  | 8.5   |
| w36                  | 1.1    | 0                    | m41   | 3.2                  | 0     | m37                  | 1.1   |
| m23                  | 1.1    | 0                    | neg.  | 0                    | 8     | neg.                 | 6     |
|                      | 0      | 1                    |       |                      |       | 1.1                  | 0     |
| neg.                 |        |                      |       |                      |       |                      |       |

Table 5 - Cont'd

| probes for codon p54 | Type B | non-B | probes for codon p82/84 | Type B | non-B | probes for codon p90 | Type B | non-B |
|----------------------|--------|-------|-------------------------|--------|-------|----------------------|--------|-------|
| w3                   | 71.3   | 48    | w91                     | 81.9   | 70    | w27                  | 50     | 2.5   |
| w34                  | 81.9   | 62    | w60                     | 2.1    | 12    | w37                  | 66.1   | 17.5  |
| w14                  | 3.2    | 18    | w111                    | 1.1    | 0     | w39                  | 7.1    | 0     |
| w19                  | 6.4    | 0     | w89                     | 1.1    | 10    | w50                  | 12.5   | 65    |
| w22                  | 4.3    | 8     | w42                     | 4.3    | 2     | w52                  | 7.1    | 2.5   |
| w26                  | 0      | 4     | m36                     | 2.1    | 0     | w69                  | 5.4    | 2.5   |
| w27                  | 0      | 4     | m67                     | 1.1    | 0     | w73                  | 5.4    | 22.5  |
| m55                  | 3.2    | 0     | m38                     | 2.1    | 2     | w79                  | 0      | 10    |
| m35                  | 14.9   | 4     | m105                    | 1.1    | 0     | m43                  | 19.6   | 5     |
| m37                  | 1.1    | 4     | m127                    | 1.1    | 0     | m56                  | 0      | 2.5   |
| neg.                 | 0      | 4     | m40                     | 14.9   | 2     | neg.                 | 3.6    | 12.5  |
|                      |        |       | m63                     | 3.2    | 2     |                      |        |       |
|                      |        |       | m101                    | 2.1    | 12    |                      |        |       |
|                      |        |       | neg.                    | 3.2    | 8     |                      |        |       |

Table 6

|        | USA  | France | U.K.  | Brazil | Spain | Luxemb. | Belgium |
|--------|------|--------|-------|--------|-------|---------|---------|
| p30    |      |        |       |        |       |         |         |
| w25    | 98.9 | 99.4   | 88.9  | 98.3   | 94.3  | 100.0   | 97.0    |
| w29    | 2.5  | 0.6    | 0.0   | 1.7    | 0.0   | 0.0     | 0.0     |
| w32    | 3.3  | 0.6    | 5.6   | 5.2    | 5.7   | 6.7     | 1.5     |
| w36    | 2.5  | 0.0    | 0.0   | 3.4    | 0.0   | 0.0     | 1.0     |
| m23    | 3.1  | 0.0    | 0.0   | 0.0    | 0.0   | 0.0     | 1.0     |
| neg.   | 0.6  | 0.6    | 5.6   | 0.0    | 0.0   | 0.0     | 1.0     |
| p46/48 |      |        |       |        |       |         |         |
| w47    | 94.2 | 80.5   | 83.3  | 89.7   | 97.1  | 73.3    | 82.9    |
| w45    | 8.6  | 15.6   | 0.0   | 1.7    | 5.7   | 6.7     | 11.1    |
| w72    | 4.2  | 0.0    | 16.7  | 0.0    | 2.9   | 13.3    | 5.0     |
| m41    | 0.0  | 0.0    | 0.0   | 10.3   | 0.0   | 13.3    | 1.0     |
| neg.   | 2.8  | 4.5    | 0.0   | 0.0    | 0.0   | 0.0     | 2.5     |
| p50    |      |        |       |        |       |         |         |
| w31    | 96.4 | 97.4   | 100.0 | 96.6   | 100.0 | 100.0   | 96.5    |
| w44    | 1.7  | 0.6    | 0.0   | 1.7    | 0.0   | 0.0     | 1.0     |
| w52    | 10.0 | 4.5    | 0.0   | 1.7    | 2.9   | 6.7     | 9.0     |
| m37    | 2.5  | 0.6    | 0.0   | 1.7    | 0.0   | 6.7     | 0.5     |
| neg.   | 3.1  | 2.6    | 0.0   | 3.4    | 0.0   | 0.0     | 1.5     |
| p54    |      |        |       |        |       |         |         |
| w34    | 96.9 | 82.5   | 97.2  | 87.9   | 100.0 | 53.3    | 89.4    |
| w3     | 84.7 | 77.9   | 94.4  | 69.0   | 82.9  | 46.7    | 76.9    |
| w14    | 3.3  | 5.8    | 0.0   | 3.4    | 11.4  | 0.0     | 6.5     |
| w19    | 9.2  | 2.6    | 0.0   | 1.7    | 2.9   | 6.7     | 5.5     |
| w22    | 2.8  | 10.4   | 0.0   | 0.0    | 5.7   | 0.0     | 2.5     |
| w26    | 0.0  | 1.3    | 0.0   | 0.0    | 0.0   | 0.0     | 0.0     |
| w27    | 0.0  | 1.9    | 0.0   | 0.0    | 0.0   | 0.0     | 0.5     |
| m55    | 0.0  | 0.0    | 0.0   | 0.0    | 0.0   | 13.3    | 0.5     |
| m35    | 1.1  | 0.0    | 2.8   | 6.9    | 0.0   | 46.7    | 3.0     |
| m37    | 0.0  | 0.0    | 0.0   | 0.0    | 0.0   | 13.3    | 0.0     |
| neg.   | 0.6  | 1.3    | 0.0   | 1.7    | 0.0   | 0.0     | 2.0     |
| p82/84 |      |        |       |        |       |         |         |
| w91    | 91.6 | 93.5   | 94.4  | 77.6   | 100.0 | 73.3    | 85.9    |
| w60    | 6.4  | 2.6    | 0.0   | 1.7    | 2.9   | 13.3    | 5.5     |
| w111   | 3.6  | 0.6    | 0.0   | 1.7    | 0.0   | 0.0     | 0.5     |
| w89    | 7.0  | 1.9    | 0.0   | 3.4    | 0.0   | 0.0     | 3.0     |
| w42    | 0.6  | 0.0    | 2.8   | 1.7    | 0.0   | 0.0     | 2.0     |
| m36    | 0.3  | 0.0    | 0.0   | 0.0    | 0.0   | 0.0     | 0.0     |
| m67    | 0.0  | 0.0    | 0.0   | 0.0    | 0.0   | 0.0     | 0.5     |

Table 6 - Cont'd

|      |      |        |      |        |       |         |     |
|------|------|--------|------|--------|-------|---------|-----|
| m38  | 0.0  | 0.0    | 0.0  | 0.0    | 0.0   | 6.7     | 0.0 |
| m105 | 0.0  | 0.0    | 0.0  | 0.0    | 0.0   | 0.0     | 0.0 |
| m127 | 0.0  | 0.0    | 0.0  | 0.0    | 0.0   | 0.0     | 0.0 |
| m40  | 2.8  | 0.0    | 8.3  | 3.4    | 5.7   | 46.7    | 0.0 |
| m63  | 0.3  | 0.0    | 0.0  | 1.7    | 2.9   | 13.3    | 0.5 |
| m101 | 1.9  | 4.5    | 0.0  | 3.4    | 0.0   | 6.7     | 4.0 |
| neg. | 2.5  | 3.9    | 0.0  | 13.8   | 0.0   | 6.7     | 5.0 |
|      |      |        |      |        |       |         |     |
| p90  | USA  | France | U.K. | Brazil | Spain | Belgium |     |
| w27  | 51.1 | 45.5   | 34.3 | 47.7   | 52.8  | 25.7    |     |
| w37  | 91.9 | 73.4   | 80.0 | 81.8   | 88.9  | 55.2    |     |
| w39  | 0.0  | 0.0    | 0.0  | 0.0    | 0.0   | 2.9     |     |
| w50  | 2.6  | 23.8   | 2.9  | 13.6   | 11.1  | 21.9    |     |
| w52  | 8.4  | 11.2   | 5.7  | 6.8    | 13.9  | 4.8     |     |
| w69  | 5.2  | 1.4    | 5.7  | 2.3    | 0.0   | 3.8     |     |
| w73  | 6.1  | 9.1    | 0.0  | 0.0    | 8.3   | 6.7     |     |
| w79  | 7.1  | 11.2   | 8.6  | 9.1    | 5.6   | 5.7     |     |
| m43  | 1.9  | 0.0    | 11.4 | 0.0    | 0.0   | 8.6     |     |
| m56  | 0.3  | 1.4    | 0.0  | 0.0    | 0.0   | 0.0     |     |
| neg. | 1.0  | 0.0    | 0.0  | 0.0    | 0.0   | 18.1    |     |

Table 7

|          |     |     |     |     |     |     |     |     |     | Tm  | length | Seq | ID  |     |     |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--------|-----|-----|-----|-----|
| pc50w5   | AGG | GGG | AAT | TGG | AGG | TTT | TA  |     |     | 20  |        | 511 |     |     |     |
|          | 26  | 27  | 28  | 29  | 30  | 31  | 32  | 33  | 34  | 35  |        |     |     |     |     |
| pc30w25  | ACA | GGA | GCA | GAT | GAT | ACA | GTA | TTA | GAA | GAA |        |     |     |     |     |
|          |     |     | GCA | GAT | GAT | ACA | GT  |     |     |     | 40     | 14  | 31  |     |     |
| pc30w29  |     |     | A   | GCG | GAT | GAT | ACA |     |     |     | 36     | 13  | 35  |     |     |
| pc30w32  |     |     |     | GCA | GAT | GAC | ACA | GT  |     |     | 42     | 14  | 38  |     |     |
| pc30w36  |     |     |     | GCA | GAC | GAT | ACA | GG  |     |     | 40     | 14  | 42  |     |     |
| pc30m23  |     |     |     | A   | GCA | GAT | AAT | ACA | GT  |     | 40     | 15  | 29  |     |     |
|          | 44  | 45  | 46  | 47  | 48  | 49  | 50  | 51  | 52  |     |        |     |     |     |     |
| pc48w37  | CCA | AAA | ATG | ATA | GGG | GGA | ATT | GGA | GGT |     |        |     |     |     |     |
|          |     |     |     | ATG | ATA | GGG | GGA | ATT |     |     | 15     |     | 512 |     |     |
| pc48w47  |     |     | AAA | ATG | ATA | GGG | GGA |     |     |     | 42     | 15  | 93  |     |     |
| pc48w73  |     |     | A   | AGA | ATG | ATA | GGG | G   |     |     | 14     |     | 513 |     |     |
| pc48w45  |     |     |     | AAA | ATG | ATA | GGA | GGA | ATT |     | 42     | 18  | 91  |     |     |
| pc48w72  |     |     |     | A   | AAA | ATA | ATA | GGG | GGA |     | 42     | 16  | 120 |     |     |
| pc48m41  |     |     |     |     | ATG | ATA | GTG | GGA | ATT |     | 40     | 15  | 87  |     |     |
|          | 48  | 49  | 50  | 51  | 52  | 53  | 54  |     |     |     |        |     |     |     |     |
| pc50w31  | GGG | GGA | ATT | GGA | GGT | TTT | ATC |     |     |     | 42     | 15  | 151 |     |     |
| pc50w44  |     |     |     | GGA | ATT | GGA | GGT | TTT |     |     | 42     | 14  | 164 |     |     |
| pc50w52  |     |     |     | GA  | ATT | GGA | GGC | TTG |     |     | 14     |     | 172 |     |     |
| pc50m37  | GGG | GGA | GTT | GGA |     |     |     |     |     |     | 40     | 12  | 157 |     |     |
|          | 51  | 52  | 53  | 54  | 55  | 56  | 57  | 58  |     |     |        |     |     |     |     |
| pc54w34  | GGA | GGT | TTT | ATC | AAA | GTA | AGA | CAG |     |     | 42     | 16  | 212 |     |     |
| pc54w14  |     |     | GA  | GGT | TTT | ATC | AAA | GT  |     |     | 42     | 16  | 189 |     |     |
| pc54w19  |     |     |     | GGT | TTT | ATC | AAG | GTA | A   |     | 42     | 16  | 194 |     |     |
| pc54w22  |     |     |     | A   | GGC | TTT | ATC | AAA | GTA |     | 42     | 17  | 197 |     |     |
| pc54w26  |     |     |     | GA  | GGT | TTT | ATT | AAA | GTA |     | 42     | 16  | 202 |     |     |
| pc54w27  |     |     |     |     | GA  | GGT | TTC | ATT | AAG | GTA | 40     | 16  | 204 |     |     |
| pc54m35  |     |     |     |     |     | GGT | TTT | ATT | AAG | GTA | 40     | 15  | 213 |     |     |
| pc54m37  |     |     |     |     |     | GGT | TTT | GTC | AAA | GTA | 42     | 15  | 215 |     |     |
| pc54m55  |     |     |     |     |     | A   | GGT | TTT | GTC | AGA | GT     |     | 15  | 516 |     |
|          | 78  | 79  | 80  | 81  | 82  | 83  | 84  | 85  | 86  | 87  |        |     |     |     |     |
| pc82w91  | GGA | CCT | ACA | CCT | GTC | AAC | ATA | ATT | GGA | AGA |        |     |     |     |     |
| pc82w60  |     |     |     |     | ACA | CCT | GTC | AAC | ATA | A   | 44     | 16  | 318 |     |     |
| pc82w111 |     |     |     |     |     | CA  | CCT | GTC | AAT | ATA | ATG    | 42  | 17  | 287 |     |
| pc82w89  |     |     |     |     |     | A   | CCG | GTC | AAC | ATA | ATT    | 44  | 16  | 338 |     |
| pc82m101 |     |     |     |     |     | ACA | CCT | GTT | AAC | ATA | AG     | 42  | 17  | 316 |     |
| pc82w42  |     |     |     |     |     |     | ACA | CCT | ATC | AAC | ATA    | AT  |     | 17  | 517 |
| pc82m38  |     |     |     |     |     |     | CA  | CCT | GTC | AAC | GTA    |     | 42  | 14  | 269 |
| pc82m105 |     |     |     |     |     |     | ACA | CCT | TTC | AAC | ATA    |     | 40  | 15  | 265 |
| pc82m127 |     |     |     |     |     |     | ACG | CCC | TTC | AAC | ATA    |     | 44  | 15  | 332 |
|          |     |     |     |     |     |     | CA  | CCT | TTC | AAC | GTA    | ATG | 44  | 17  | 354 |

Table 7 - Cont'd

|          |     |     |     |     |     |     |     |     |     |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| pc82m40  | ACA | CCT | GCC | AAC | ATA | 44  | 15  | 267 |     |
| pc82m63  | CA  | CCT | GCC | AAT | ATA | 42  | 16  | 290 |     |
| pc82m36  | ACA | CCT | ACC | AAC | ATA | 15  | 518 |     |     |
| pc82m67  | ACA | CCT | ACC | AAC | GT  | 14  | 519 |     |     |
|          | 86  | 87  | 88  | 89  | 90  | 91  | 92  | 93  | 94  |
|          | GGA | AGA | AAT | CTG | TTG | ACT | CAG | ATT | GGT |
| pc90w27  | AAT | CTG | TTG | ACT | CA  |     | 38  | 14  | 384 |
| pc90w37  | T   | CTG | TTG | ACT | CAG | AT  |     | 15  | 514 |
| pc90w39  | GA  | GTC | AAC | AGA | GTT | C   |     | 15  | 515 |
| pc90w50  | AAT | ATG | TTG | ACT | CAG |     | 40  | 15  | 407 |
| pc90w52  | AAT | TTG | TTG | ACT | CAG |     | 40  | 15  | 409 |
| pc90w69  | GA  | AAC | CTG | TTG | ACT |     | 40  | 14  | 426 |
| pc90w73  | TG  | TTG | ACA | CAG | CTT | G   | 44  | 15  | 430 |
| pc90w79  | TG  | TTG | ACC | CAG | ATT | G   | 44  | 15  | 436 |
| pc90m138 | GTC | ATC | AGA | TTT | CT  |     |     | 14  | 510 |
| pc90m56  | AAT | ATG | ATG | ACC | CAG |     | 42  | 15  | 413 |

## CLAIMS

5 1. Method for determining the susceptibility to antiviral drugs of HIV viruses in a biological sample, with said method comprising:

- if need be, releasing, isolating or concentrating the polynucleic acids present in the sample;
- if need be amplifying the relevant part of the protease gene of HIV with at least one suitable primer pair;
- hybridizing the polynucleic acids of step a) or b) with at least one of the following probes:  
probes specifically hybridizing to a target sequence comprising codon 30;  
probes specifically hybridizing to a target sequence comprising codon 46 and/or 48;  
probes specifically hybridizing to a target sequence comprising codon 50;  
probes specifically hybridizing to a target sequence comprising codon 54;  
probes specifically hybridizing to a target sequence comprising codon 82 and/or 84;  
probes specifically hybridizing to a target sequence comprising codon 90;  
or the complement of said probes;  
further characterized in that said probes specifically hybridize to any of the target sequences presented in figure 1, or to the complement of said target sequences;
- inferring from the result of step c) whether or not a mutation giving rise to drug resistance is present in any of said target sequences.

25 2. Method according to claim 1, further characterized in that said polynucleic acids of step a) or b) hybridize with at least two of the said probes, or to the complement of said probes.

3. Method according to claim 2, further characterized in that said probes are chosen from the following list: seq id no 7 to seq id no 477, seq id no 510 to seq id no 519 or the complement of said probes.

30 4. Method according to any of claims 1 to 3, further characterized in that said primer pair is chosen from the following primers: seq id no 3, seq id no 503, seq id no 504, seq id no 4, seq id no 506, seq id no 507, seq id no 508 and seq id no 509.

35 5. Method according to any of claims 1 to 3, further characterized in that:

step b) comprises amplifying a fragment of the protease gene with at least one 5'-primer specifically hybridizing to a target sequence located at nucleotide position 210 to 260 of the protease gene, in combination with at least one suitable 3'-primer, and

5 step c) comprises hybridizing the polynucleic acids of step a) or b) with at least one of the probes specifically hybridizing to a target sequence or its complement, comprising codon 90.

6. Method according to any of claims 1 to 3, further characterized in that:

step b) comprises amplifying a fragment of the protease gene with at least one 3'-primer specifically hybridizing to a target sequence located at nucleotide position 253 (codon 85) to position 300, in combination with at least one suitable 5'-primer, and

10 step c) comprises hybridizing the polynucleic acids of step a) or b) with at least one of the probes specifically hybridizing to a target sequence or its complement, comprising any of codons 30, 46, 48, 50, 52, 54, 82 and 84.

15 7. Method according to claim 5, further characterized in that the 5'-primer is seq id 5 and the 3'-primer is one primer or a combination of primers chosen from the following primers: seq id no 4, seq id no 506, seq id no 507, seq id no 508 and seq id no 509.

20 8. Method according to claim 6, further characterized in that the 5'-primer is one primer or a combination of primers chosen from the following primers: seq id no 3, seq id no 503, seq id no 504 and the 3'-primer is seq id no 6.

9. A probe as defined in any of claims 1 to 3, for use in a method for determining the susceptibility to antiviral drugs of HIV viruses in a biological sample.

25 10. A nucleic acid comprising a nucleotide sequence represented by any of the following SEQ ID numbers: SEQ ID NO 478, SEQ ID NO 479, SEQ ID NO 480, SEQ ID NO 481, SEQ ID NO 482, SEQ ID NO 483, SEQ ID NO 484, SEQ ID NO 485, SEQ ID NO 486, SEQ ID NO 487, SEQ ID NO 488, SEQ ID NO 489, SEQ ID NO 490, SEQ ID NO 491, SEQ ID NO 492, SEQ 30 ID NO 493, SEQ ID NO 494, SEQ ID NO 495, SEQ ID NO 496, SEQ ID NO 497, SEQ ID NO 498, SEQ ID NO 499 and SEQ ID NO 500; or a fragment thereof, wherein said fragment consists of at least two contiguous nucleotides and contains at least one polymorphic nucleotide.

35 11. A primer as defined in any of claims 4 to 8, for use in a method for determining the susceptibility to antiviral drugs of HIV viruses in a biological sample.

12. A diagnostic kit enabling a method for determining the susceptibility to antiviral drugs of HIV viruses in a biological sample, with said kit comprising:

- 5 a) when appropriate, a means for releasing, isolating or concentrating the polynucleic acids present in said sample;
- b) when appropriate, at least one of the primers of any of claims 4 to 6;
- c) at least one of the probes of any of claims 1 to 3, possibly fixed to a solid support;
- d) a hybridization buffer, or components necessary for producing said buffer;
- e) a wash solution, or components necessary for producing said solution;
- 10 f) when appropriate, a means for detecting the hybrids resulting from the preceding hybridization;
- h) when appropriate, a means for attaching said probe to a solid support.

**Figure 1**

### Codon 30

| 26  | 27  | 28  | 29  | 30  | 31  | 32  | 33  | 34  | 35  |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| ACA | GGA | GCA | GAT | GAT | ACA | GTA | TTA | GAA | GAA |
| G   | G   | C   | A   |     | A   | G   |     |     |     |
|     |     |     |     | C   |     | G   | G   |     |     |
|     |     |     |     |     |     | C   |     |     |     |
|     |     |     |     |     |     | C   |     |     |     |
|     |     |     |     |     |     |     | G   |     |     |

### Codon 46/48

|     |     |     |     |     |     |     |     |     |     |     |   |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|---|
| 44  | 45  | 46  | 47  | 48  | 49  | 50  | 51  | 52  | 53  | 54  |   |
| CCA | AAA | ATG | ATA | GGG | GGA | ATT | GGA | GGT | TTT | ATC |   |
| G   | T   | G   | T   | A   | G   |     | G   | GG  |     |     |   |
| G   | A   |     | A   | G   | G   |     |     | G   |     |     | G |

### Codon 50

### Codon 54

|     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|
| 51  | 52  | 53  | 54  | 55  | 56  | 57  | 58  |
| GGA | GGT | TTT | ATC | AAA | GTA | AGA | CAG |
| G   | C   | C   | G   | G   | C   | A   | G   |
| G   | A   | C   |     | G   |     | G   | A   |
| T   |     |     |     |     |     |     |     |
| GC  |     |     |     |     |     |     |     |

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**Figure 1 - Cont'd**

Codon 82/84

| 78  | 79  | 80  | 81  | 82  | 83  | 84  | 85  | 86  | 87  |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| GGA | CCT | ACA | CCT | GTC | AAC | ATA | ATT | GGA | AGA |
| A   | T   | G   | T   | C   | G   |     | G   |     |     |
| G   | T   | C   | A   |     | T   |     |     | G   |     |
|     |     |     |     |     |     |     |     | GG  |     |
|     |     | G   | A   | C   |     |     |     |     |     |
|     |     |     |     |     | T   |     |     |     | C   |
|     |     |     |     |     | AC  |     |     |     |     |
|     |     |     |     |     | TC  |     |     |     |     |

### Codon 90

| 86  | 87  | 88  | 89  | 90  | 91  | 92  | 93  | 94  |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| GGA | AGA | AAT | CTG | TTG | ACT | CAG | ATT | GGT |
| C   | C   | A   | A   | C   | A   | C   | C   | G   |
| A   | C   | T   | C   | A   | A   | G   | C   | C   |
| G   | C   |     | A   |     |     | G   |     | A   |
|     |     | A   | AA  |     |     | A   |     |     |
|     |     | A   | A   |     |     | GG  |     |     |
|     |     |     |     |     |     | C   | G   |     |

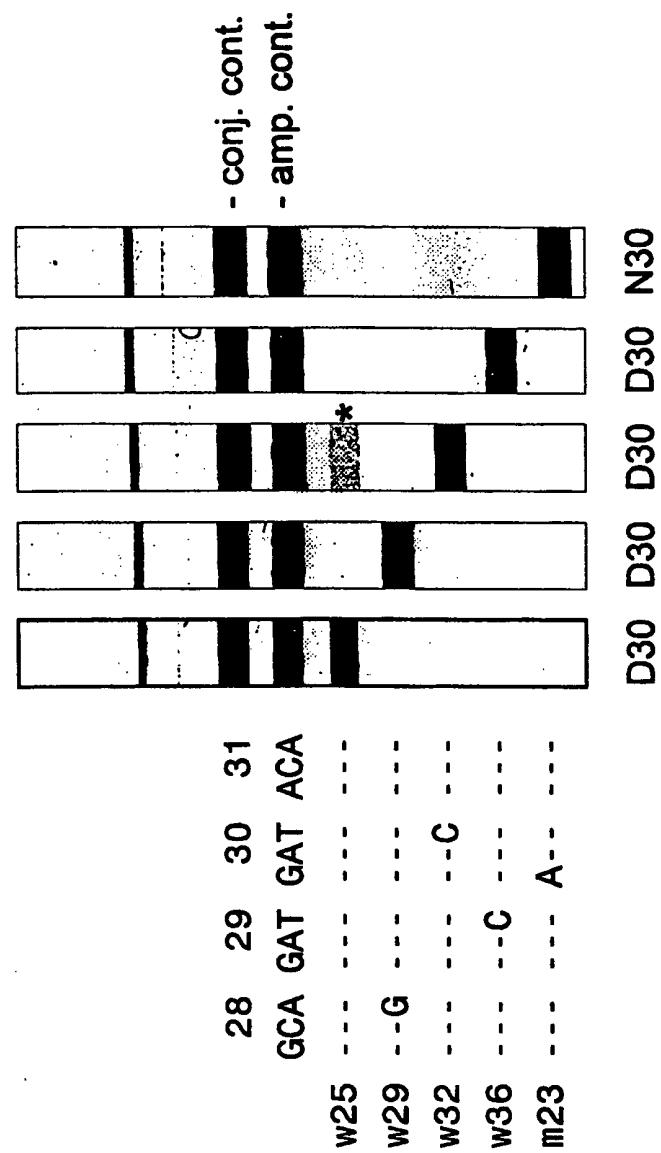


Figure 2A

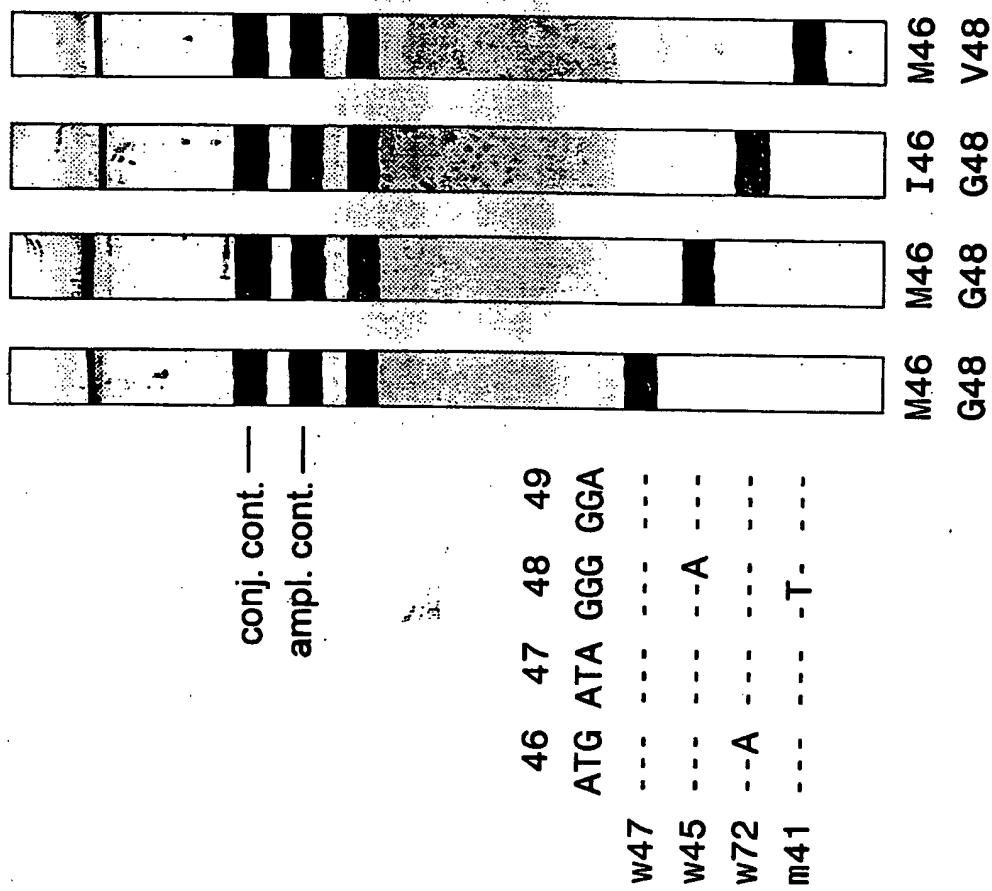


Figure 2B

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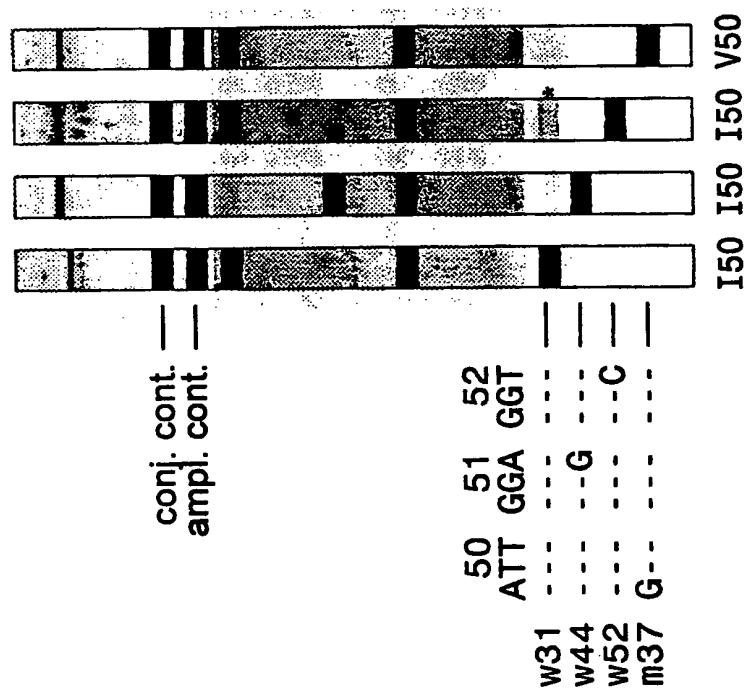


Figure 2C

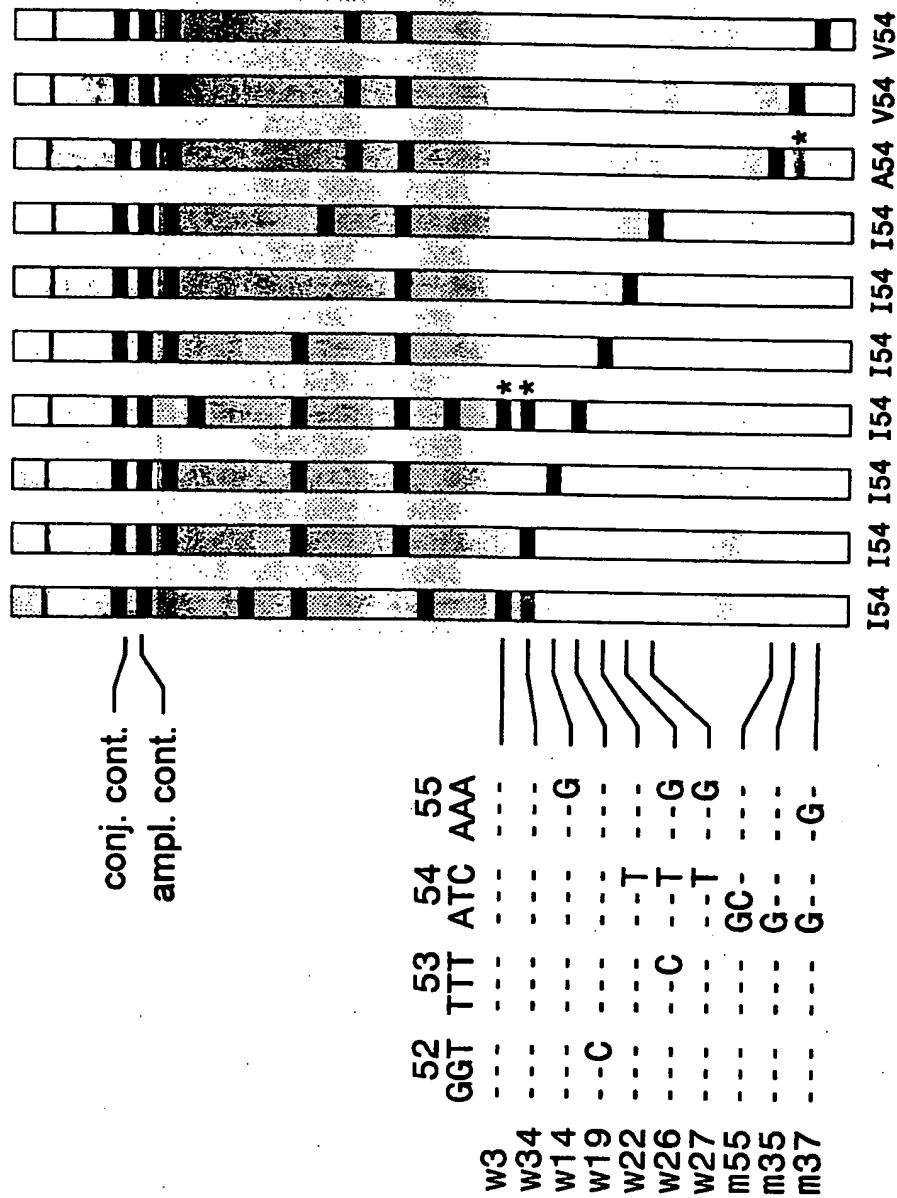
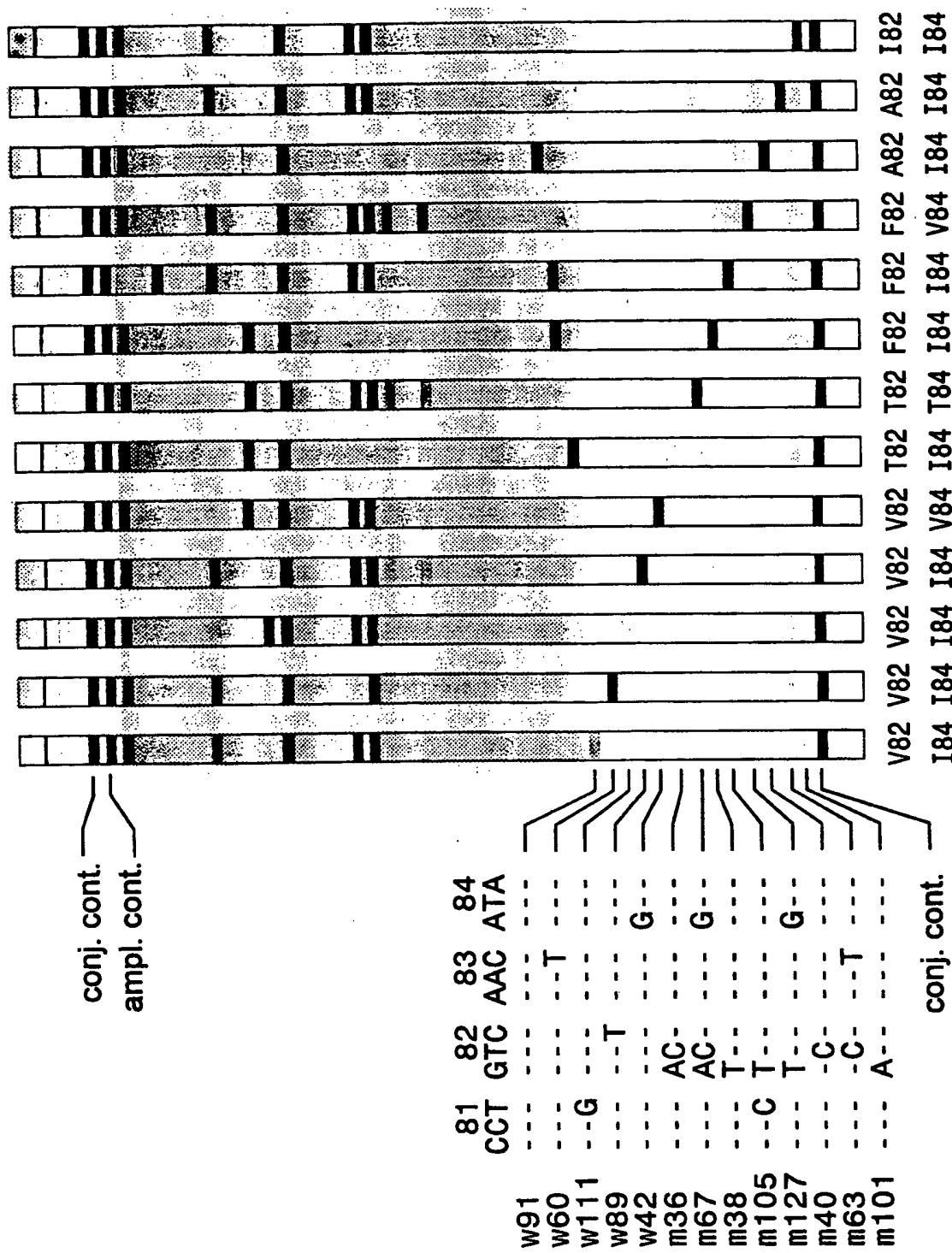


Figure 2D



**Figure 2E**

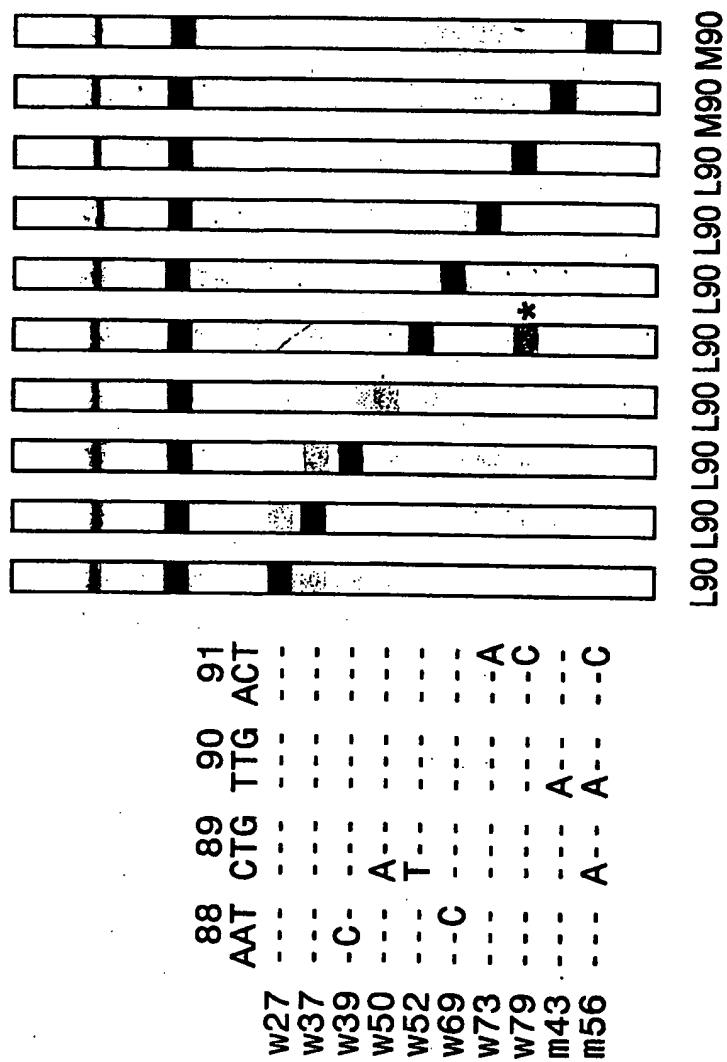
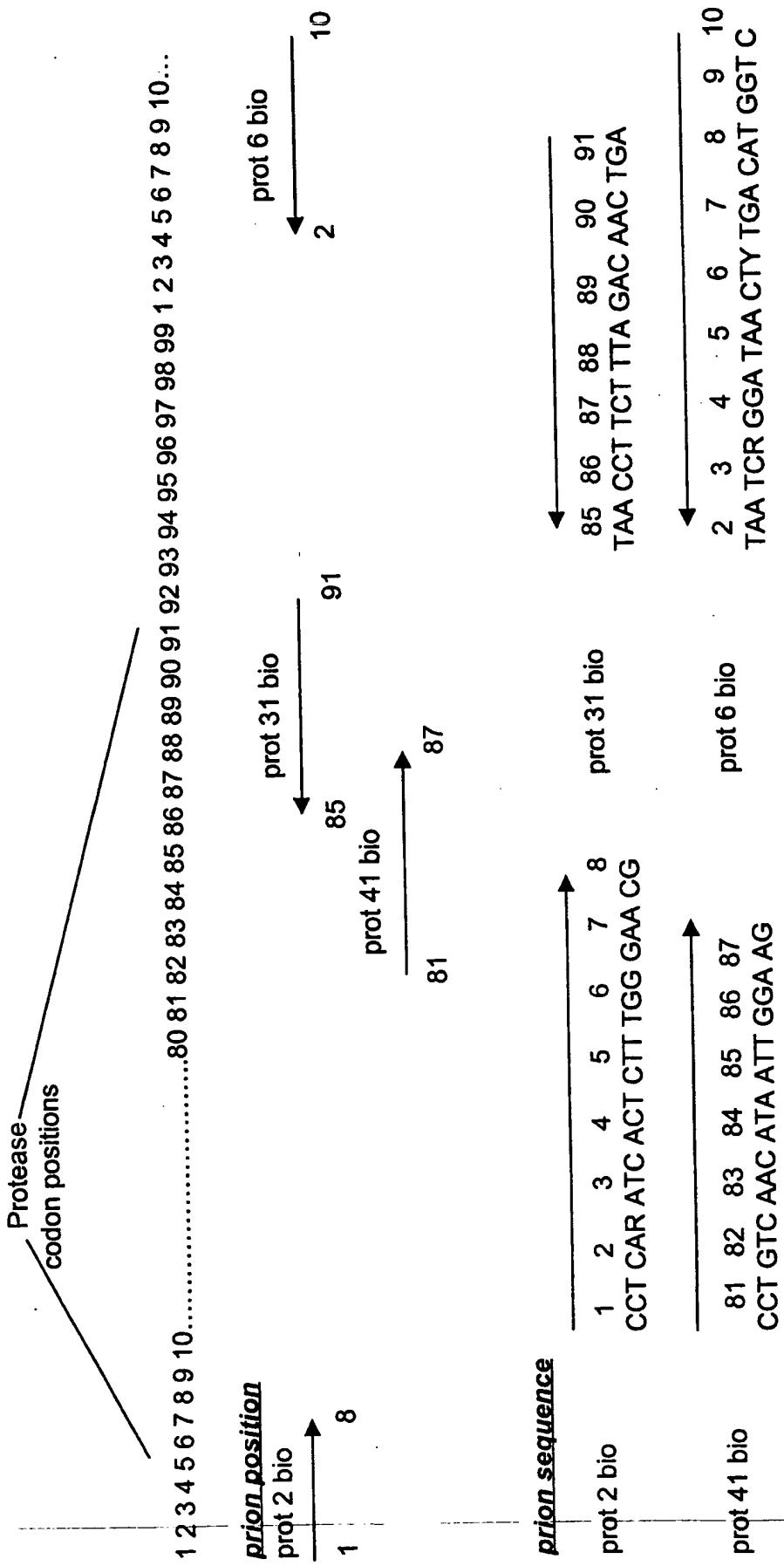


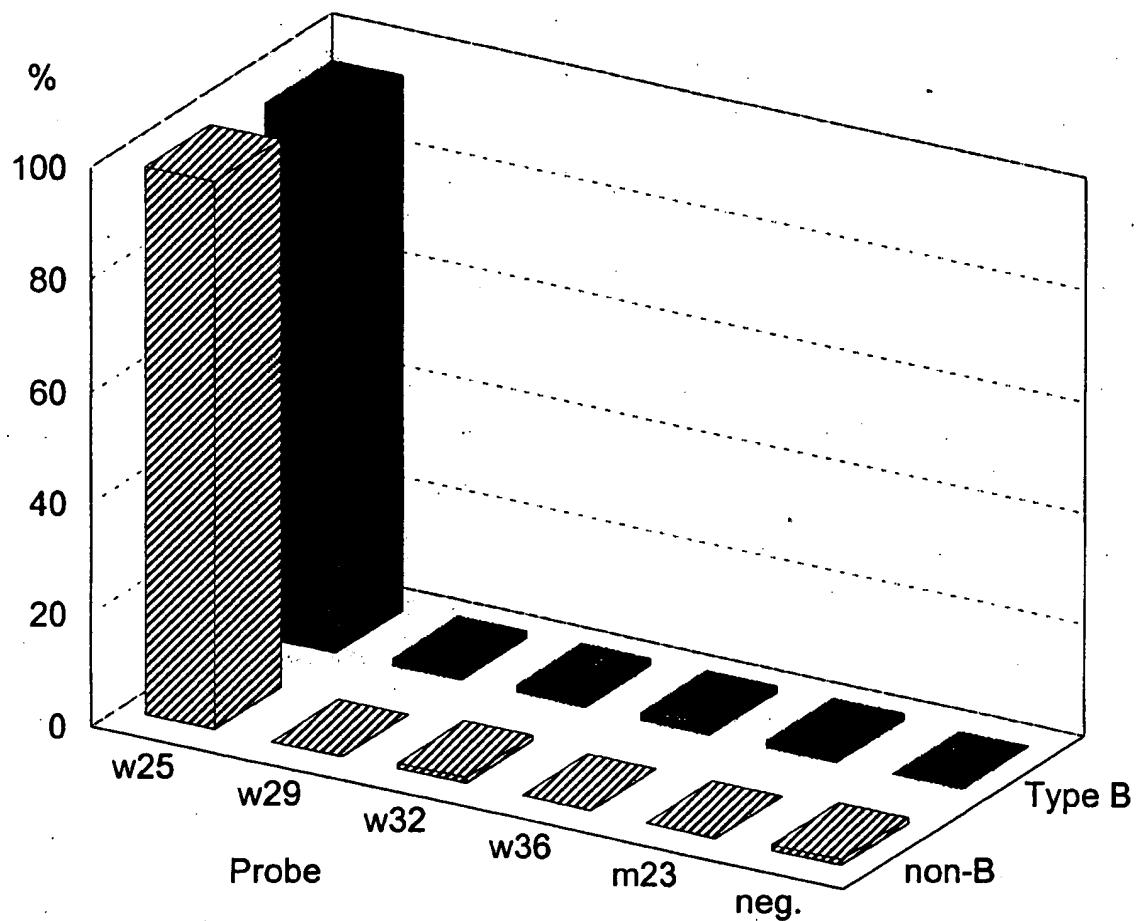
Figure 2F

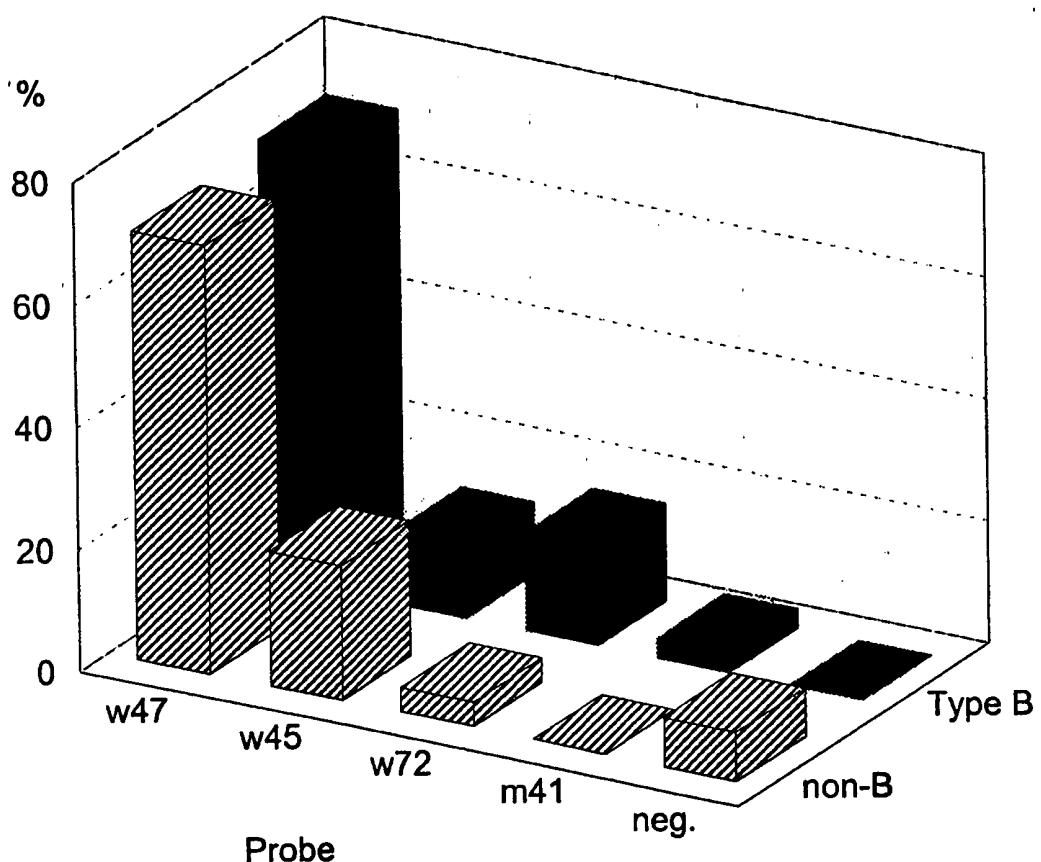
9/21

**Figure 3**

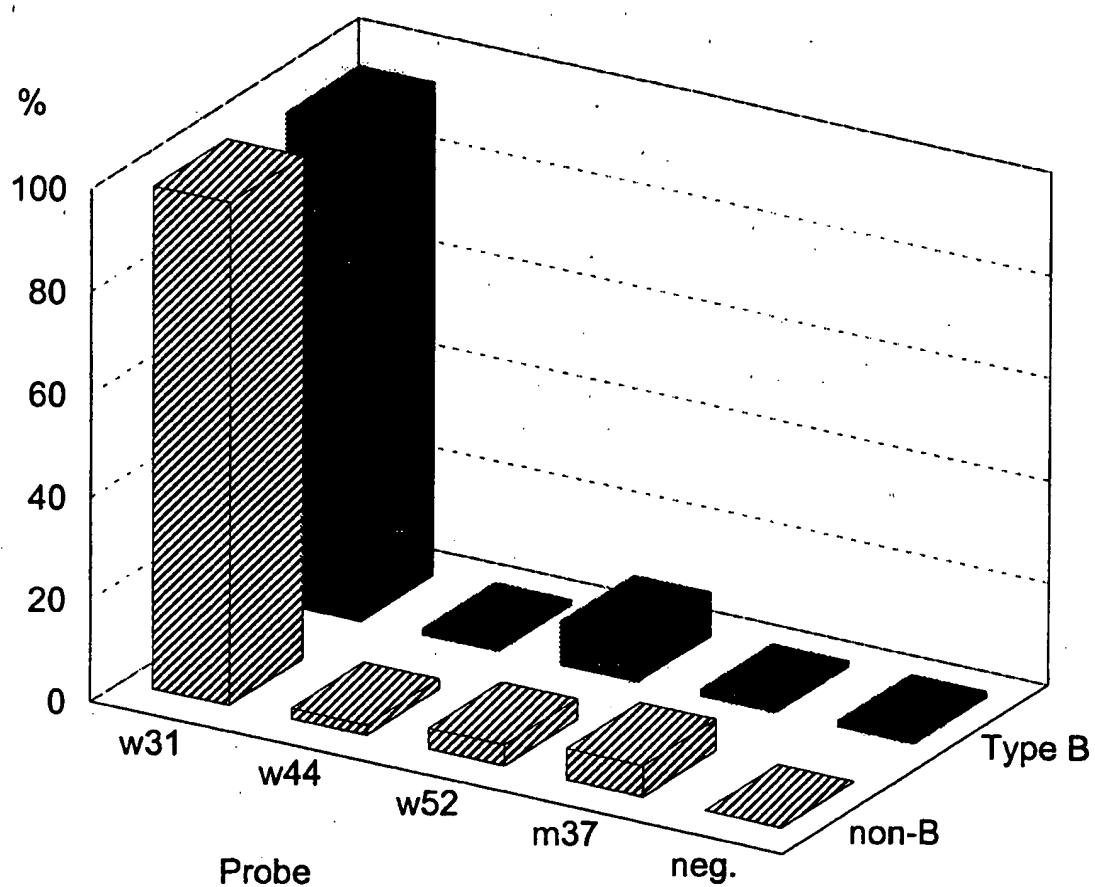


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**Figure 4A**

**Figure 4B**

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**Figure 4C**

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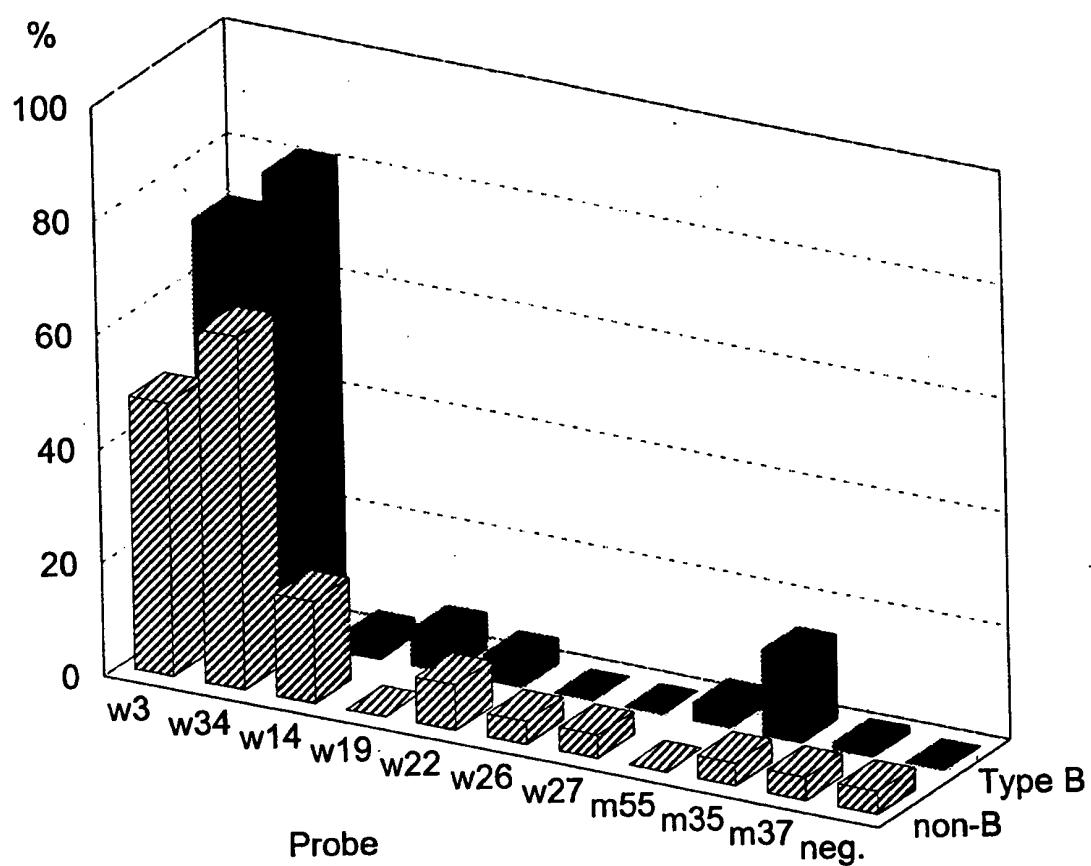
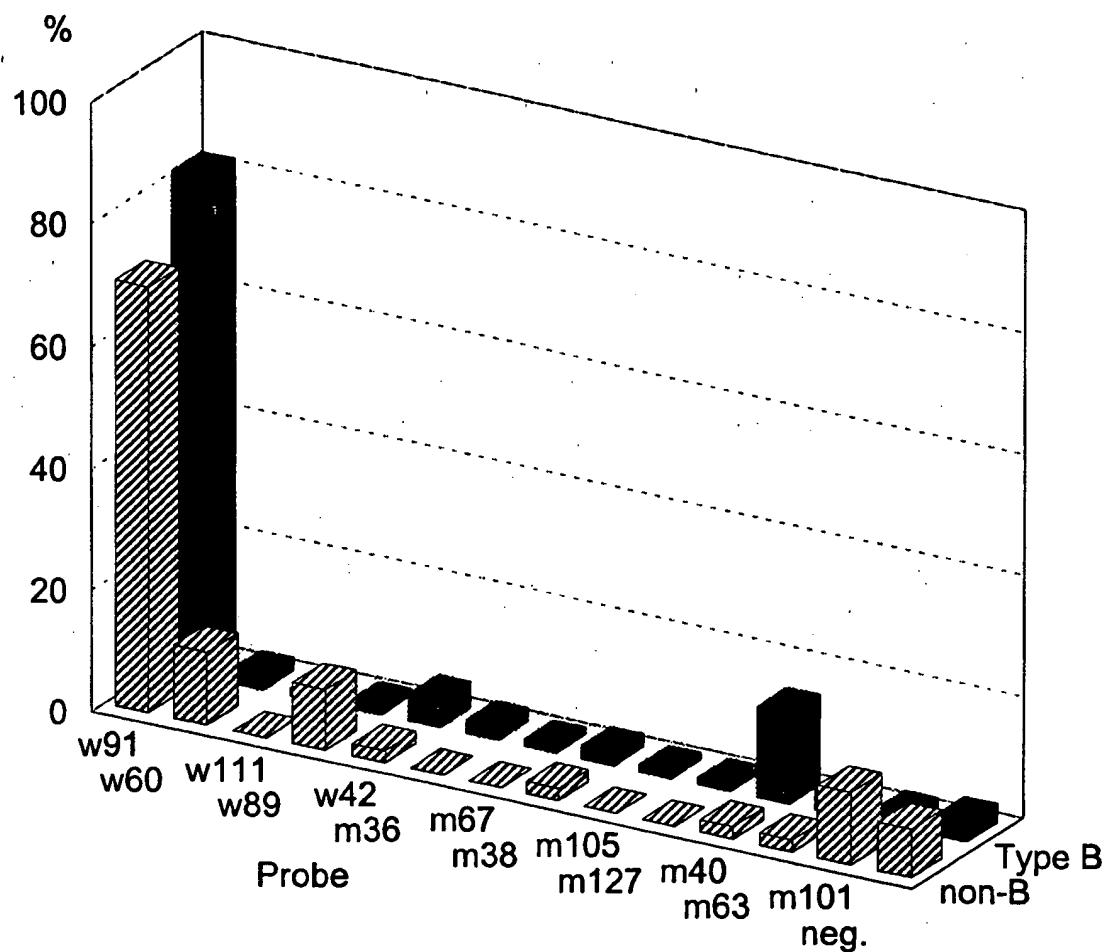
**Figure 4D**

Figure 4E



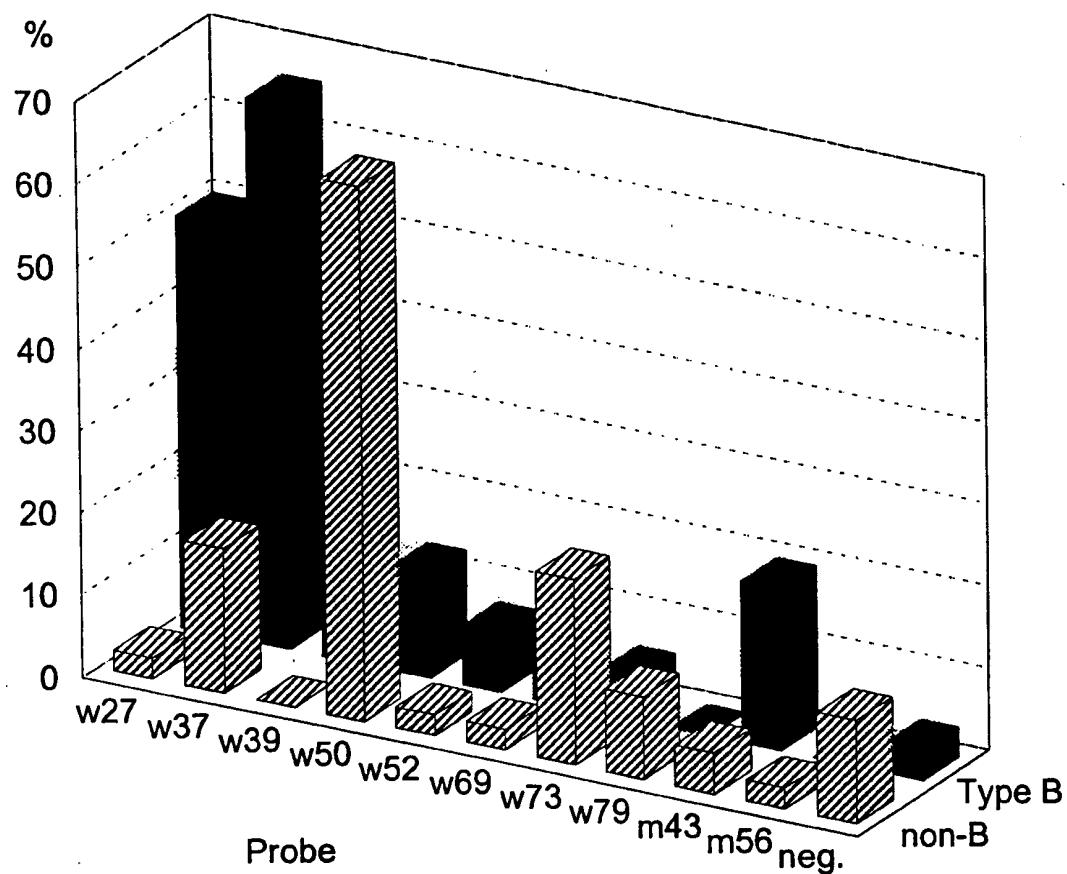
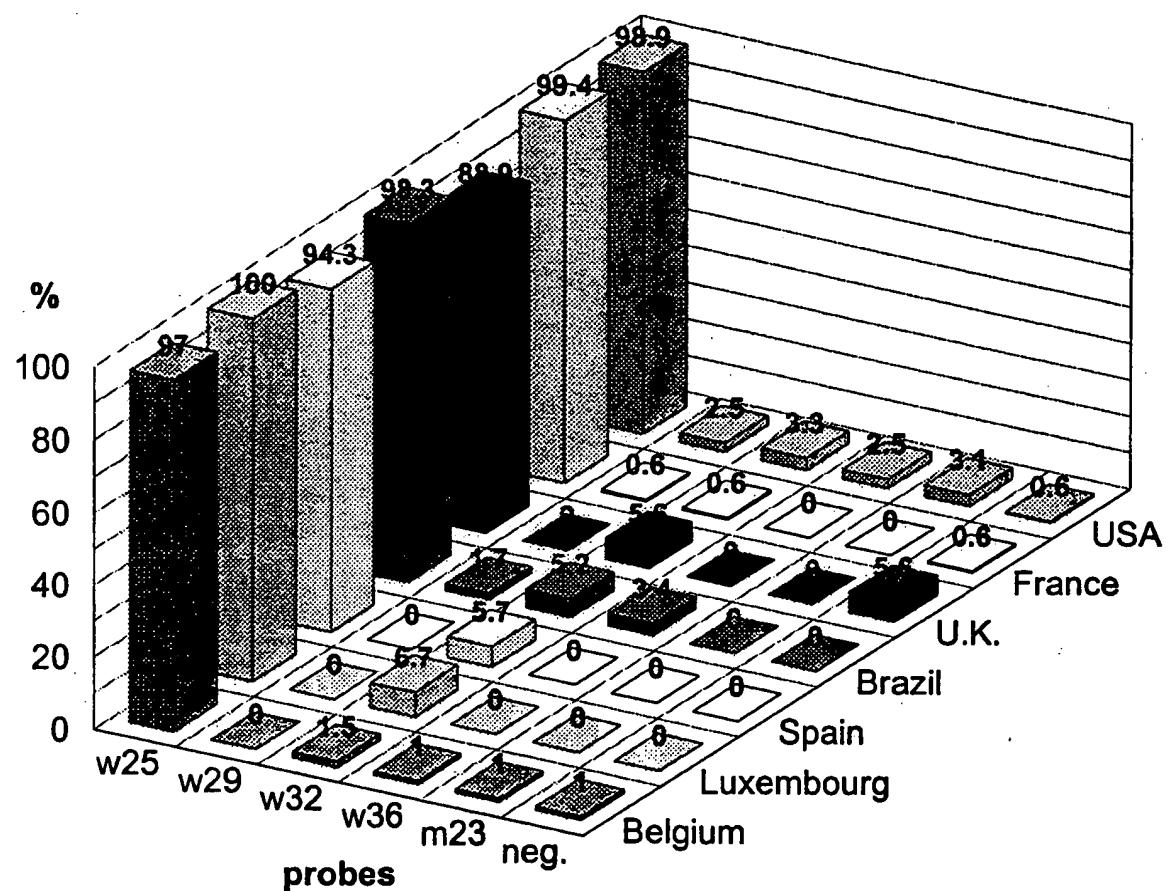
**Figure 4F**

Figure 5A



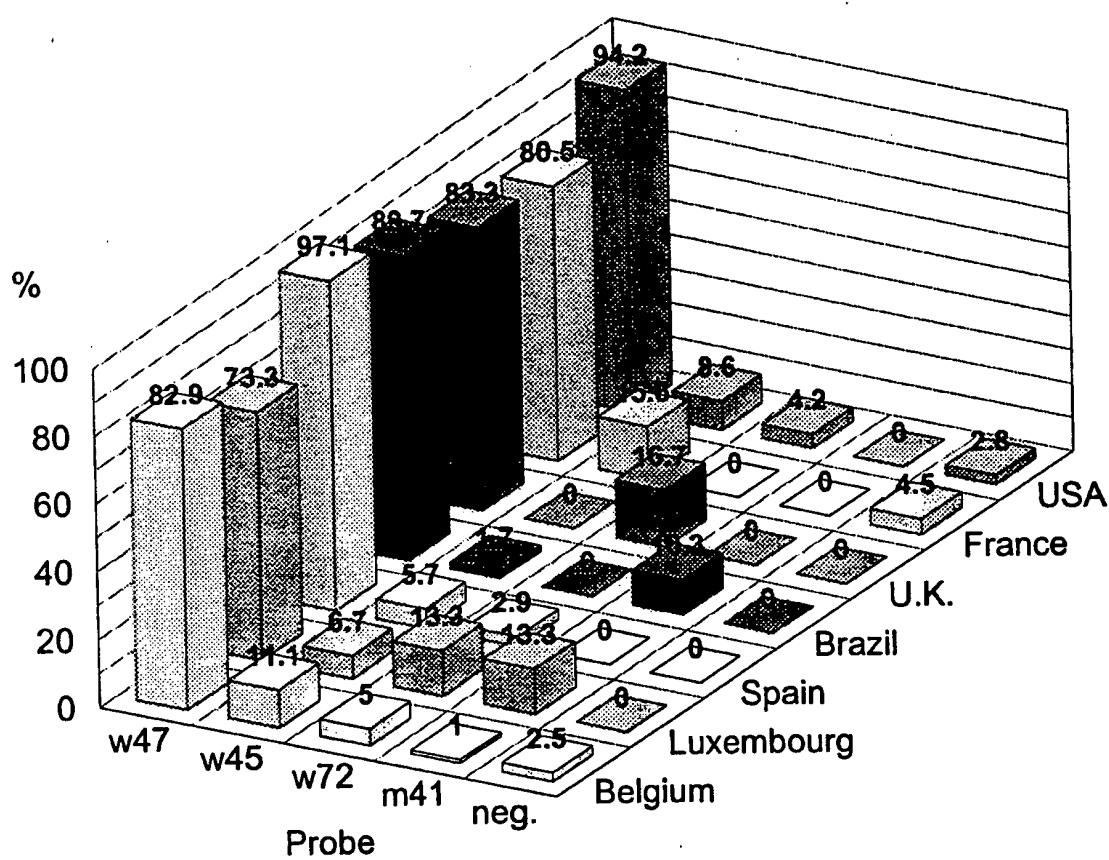
**Figure 5B**

Figure 5C

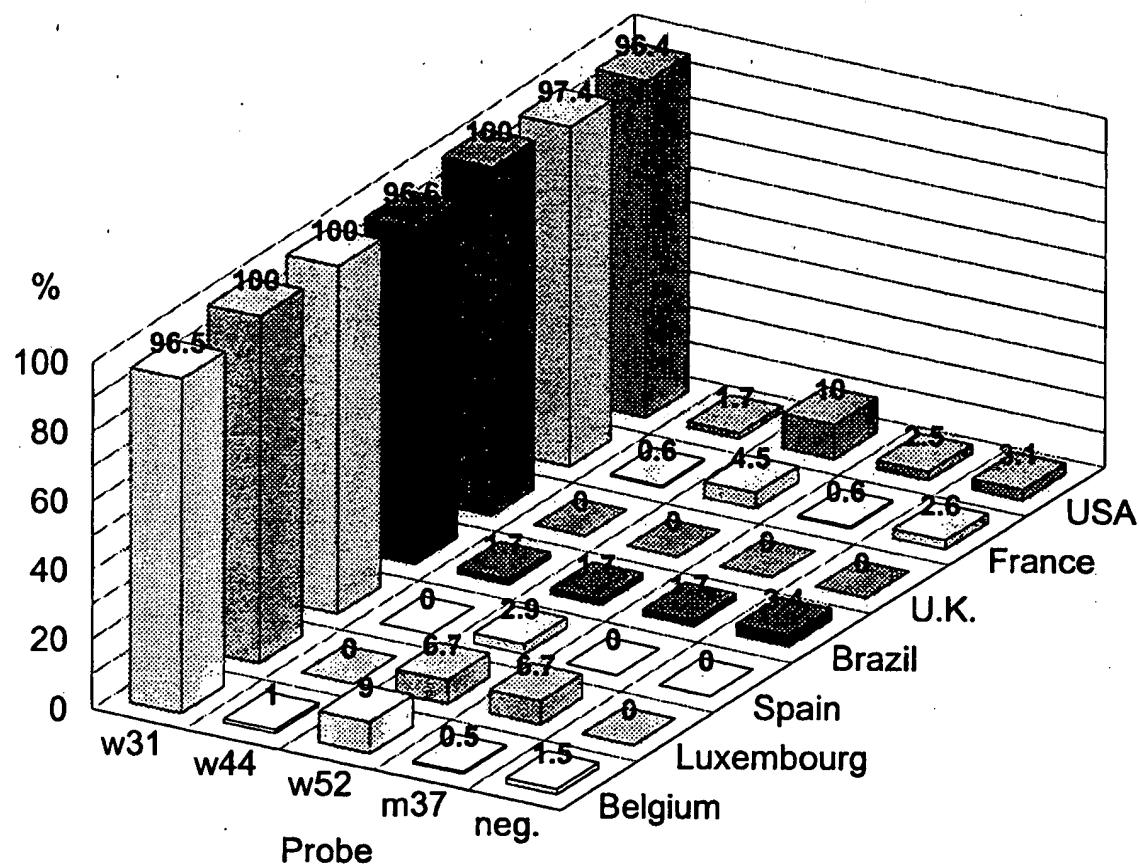


Figure 5D

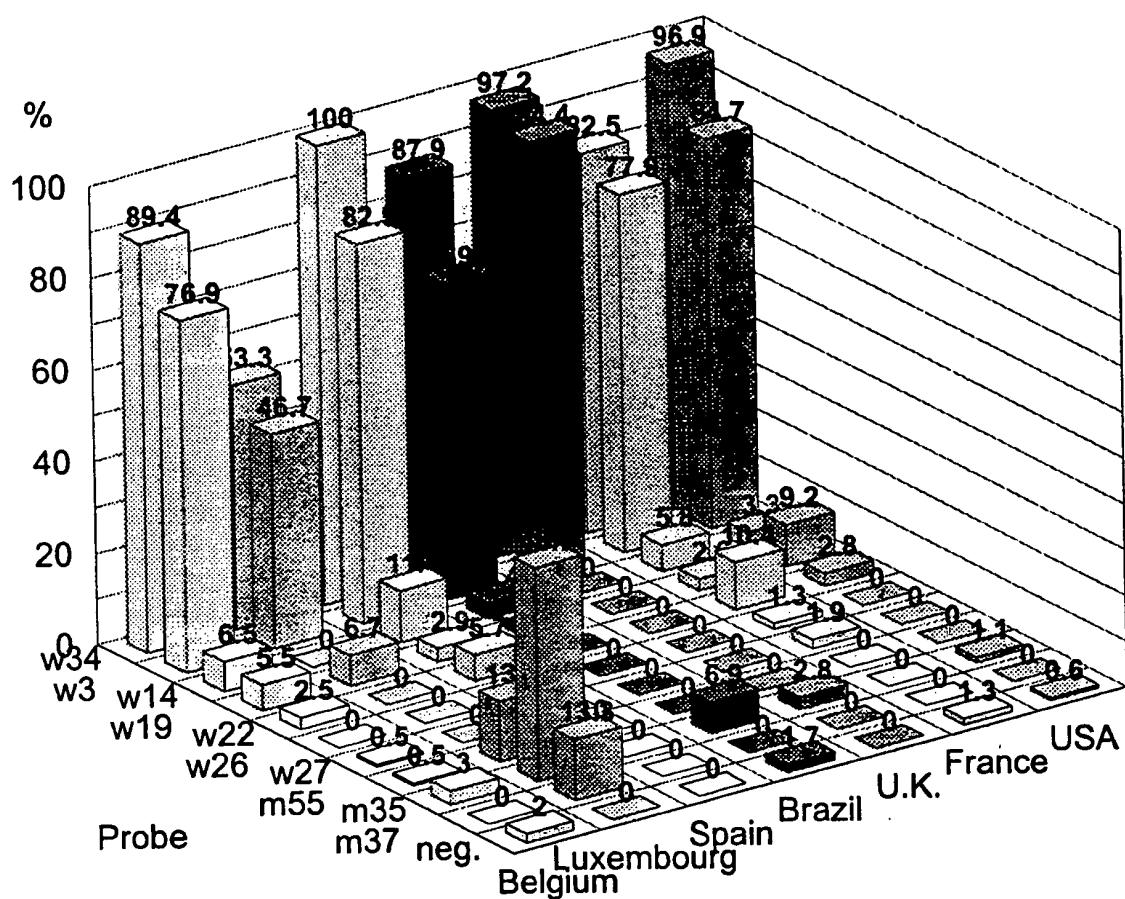


Figure 5E

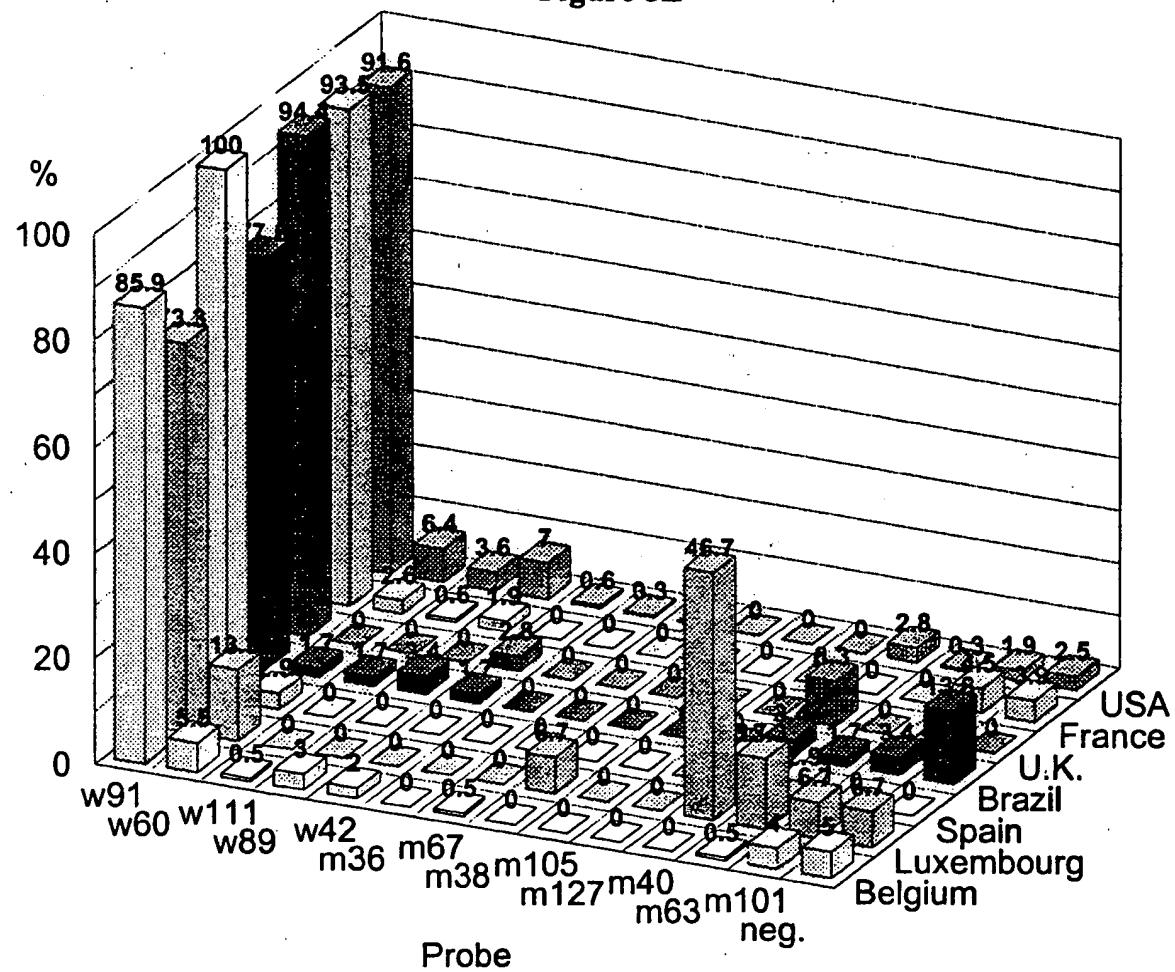
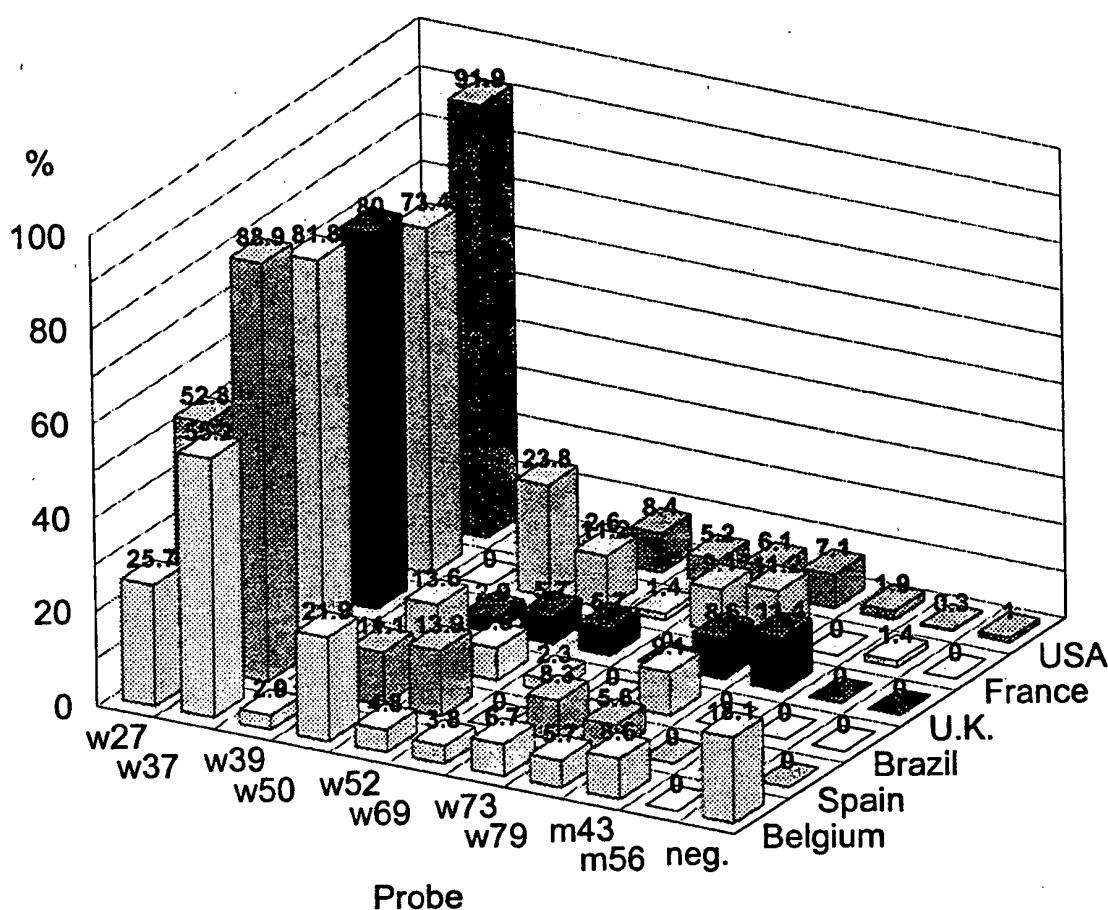


Figure 5F





## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

|  |   |  |   |
|--|---|--|---|
| (51) International Patent Classification 6:<br><br>C12Q 1/70 |   | A3   | (11) International Publication Number: <b>WO 99/67428</b><br><br>(43) International Publication Date: 29 December 1999 (29.12.99) |
| (21) International Application Number:                       | PCT/EP99/04317  | (81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). |   |
| (22) International Filing Date:                              | 22 June 1999 (22.06.99)   |  |   |
| (30) Priority Data:  | 98870143.9 24 June 1998 (24.06.98)  | EP   |   |
| (71) Applicant (for all designated States except US):        | INNOGENETICS N.V. [BE/BE]; Industriepark Zwijnaarde 7, P.O. Box 4, B-9052 Ghent (BE). |  |   |
| (72) Inventor; and   |   |  |   |
| (75) Inventor/Applicant (for US only):                       | STUYVER, Lieven [BE/BE]; Holestraat 8, B-9552 Herzele (BE).                           |  | Published<br>With international search report.  |
| (74) Common Representative:                                  | INNOGENETICS N.V.; Industriepark Zwijnaarde 7, P.O. Box 4, B-9052 Ghent (BE).         |  | (88) Date of publication of the international search report:<br>13 April 2000 (13.04.00)  |

(54) Title: METHOD FOR DETECTION OF DRUG-SELECTED MUTATIONS IN THE HIV PROTEASE GENE

## (57) Abstract

The present invention relates to a method for the rapid and reliable detection of drug-selected mutations in the HIV protease gene allowing the simultaneous characterization of a range of codons involved in drug resistance using specific sets of probes optimized to function together in a reverse-hybridization assay. More particularly, the present invention relates to a method for determining the susceptibility to antiviral drugs of HIV viruses in a biological sample, with said method comprising: a) if need be, releasing, isolating or concentrating the polynucleic acids present in the sample; b) if need be amplifying the relevant part of the protease gene of HIV with at least one suitable primer pair; c) hybridizing the polynucleic acids of step a) or b) with at least one of the following probes: probes specifically hybridizing to a target sequence comprising codon 30; probes specifically hybridizing to a target sequence comprising codon 46 and/or 48; probes specifically hybridizing to a target sequence comprising codon 50; probes specifically hybridizing to a target sequence comprising codon 54; probes specifically hybridizing to a target sequence comprising codon 82 and/or 84; probes specifically hybridizing to a target sequence comprising codon 90; or the complement of said probes; further characterized in that said probes specifically hybridize to any of the target sequences presented in figure (1), or the complement of said target sequences; d) inferring from the result of step c) whether or not a mutation giving rise to drug resistance is present in any of said target sequences.

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 99/04317

**A. CLASSIFICATION OF SUBJECT MATTER**  
**IPC 6 C12Q1/70**

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

**IPC 6 C12Q**

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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|----------|---|-----------------------|
| X        | <p>EASTMAN ET AL: "Genotypic changes in human immunodeficiency virus type 1 associated with loss of suppression of plasma viral RNA levels in subjects treated with ritonavir (norvir) monotherapy"<br/>         JOURNAL OF VIROLOGY, June 1998 (1998-06),<br/>         XP002129272<br/>         the whole document</p> <p>---</p> <p style="text-align: center;">-/-</p> | 1-9                   |



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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Date of the actual completion of the international search

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Date of mailing of the international search report

11/02/2000

Name and mailing address of the ISA

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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 99/04317

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| X        | LIPSHUTZ R J ET AL: "USING OLIGONUCLEOTIDE PROBE ARRAYS TO ACCESS GENETIC DIVERSITY" BIOTECHNIQUES, US, EATON PUBLISHING, NATICK, vol. 19, no. 3, 1 September 1995 (1995-09-01), pages 442-447, XP000541924 ISSN: 0736-6205 the whole document  | 1,2,9                 |
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| Y        | CORDOBA J. ET AL: "'Human immunodeficiency virus and resistance!'. VIRUS DE LA INMUNODEFICIENCIA HUMANA Y RESISTENCIAS." REVISTA ESPANOLA DE QUIMIOTERAPIA, (1998) 11/2 (152-156). , XP000867234 the whole document   | 1-9                   |
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| A        | WO 97 27332 A (INNOGENETICS NV ;STUYVER LIEVEN (BE); LOUWAGIE JOOST (BE); ROSSAU) 31 July 1997 (1997-07-31) the whole document  | 1-12                  |
| A        | WINTERS ET AL : "Human immunodeficiency virus type 1 protease genotypes and in vitro protease inhibitor susceptibilities of isolates from individuals who where switched to other protease inhibitors after long-term sequinavir treatment" JOURNAL OF VIROLOGY, vol. 22, no. 6, June 1998 (1998-06), pages 5303-5306, XP002129273 the whole document | 1-12                  |

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## INTERNATIONAL SEARCH REPORT

Int. Application No

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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|----------|--|-----------------------|
| P, X     | <p>SCHOOLMEESTER, A. (1) ET AL: "A line probe assay (LiPA) for the detection of drug-selected mutations in the HIV -1 protease gene."</p> <p>ABSTRACTS OF THE INTERSCIENCE CONFERENCE ON ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, (1998) VOL. 38, PP. 396-397. MEETING INFO.: 38TH INTERSCIENCE CONFERENCE ON ANTIMICROBIAL AGENTS AND CHEMOTHERAPY SAN DIEGO, CALIFORNIA, USA SEPTEMBER 24-27, 1998 AMER, XP000869787 abstract</p> <p>-----</p> | 1-12                  |

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP 99/04317

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
2.  Claims Nos.: 9, 10 and 12 because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
see FURTHER INFORMATION sheet PCT/ISA/210
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 9,10,12

Present claim 9 relates to a vast amount of nucleic acids so that a lack of conciseness within the meaning of Article 6 PCT arises to such an extent as to render a meaningful search of the claim impossible. Consequently the claimed nucleic acid sequences have not been searched per se.

Present claim 10 relates to an extremely large number of possible nucleic acid sequences so that a lack of clarity and conciseness within the meaning of Article 6 PCT arises to such an extent as to render a meaningful search of the claim impossible. Consequently, the search has been carried out for those parts of the claim which do appear to be clear and concise, namely the nucleic acid sequences themselves, which are specified with a sequence ID number.

Neither nucleic acids comprising these sequences nor fragments of these sequences, wherin said fragment consists of at least two contiguous nucleotides and contains at least one polymorphic nucleotide, have been searched.

Present claim 12 relates to a vast amount of nucleic acids that a lack of clarity and conciseness within the meaning of Article 6 PCT arises to such an extent as to render a meaningful search of the claim impossible. Consequently the nucleic acid sequences being part of the claimed kit have not been searched per se.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No  
PCT/EP 99/04317

| Patent document cited in search report | Publication date | Patent family member(s) |       | Publication date |
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